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PHYSIOLOGICAL ZOÖLOGY

Volume XXI APRIL 1948 Number 2

GRADIENT PATTERNS OF THE NITROPRUSSIDE REACTION IN STYLARIA FOSSULARIS

J. CHU

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THE extensive work done by Thompson and Voegtlin (1926), Murray (1926), Yaoi (1928), and White (1930) has shown in both plant and animal materials that the sulphydryl group content is generally higher in the embryonic tissues than in their corresponding specialized or adult tissues. According to Baker (1929) and Hammett (1929 and 1930), the -SH group exerts a stimulative effect upon cell proliferation. But, by the use of sulphydryl compounds, Sun (1930) and Morgulis and Green (1931) failed to obtain any positive effect either on the rate of cell proliferation or on the rate of regeneration. In *Tubifex tubifex*, however, Coldwater (1933) has demonstrated that the -SH compounds are capable of accelerating the rate of regeneration of the posterior segments, and he considered that sulphydryl is essential for regenerative growth.

The present work was undertaken to study the pattern of the distribution of the -SH group in the oligochaete worm, *Stylaria fossularis*. Special attention was paid to these worms at the time of fission and to the regenerating pieces. An approach to the physiological significance of the patterns of the -SH group reaction was also attempted.

MATERIAL AND METHOD

Having a transparent body structure, *S. fossularis* serves as a very favorable material for the study of the nitroprusside reaction. The material consisted of the worms, which were collected from their natural habitat and maintained in the original pond water for 2–3 days before use. For one series of experiments, only those worms which were undergoing one or more fissions were selected. The second series consisted of those starved worms which were obtained following the technique previously described (Chu, 1946). Tests were applied at different periods of starvation. In a third series of experiments regenerating pieces were cut at different body levels and were cultured to regenerate by the method of Chu and Pai (1944). The reactions were examined at different stages of regeneration. As controls, as well as for the study of the gradients of -SH distribution in normal worms, only animals not undergoing fission were used.

The procedure employed in the nitroprusside reaction test was essentially the same as the Coldwater modification of the methods used by previous workers (Coldwater, 1933). By means of a pipette, individual living worms were

placed on each slide. After the surrounding water had been completely sucked off, the worms were treated with 4 per cent hot acetic acid for approximately half a minute. The acid was then drained off and replaced by 2–3 drops of 5 per cent solution of sodium nitroprusside, containing saturated ammonium sulphate. After 1 minute had been allowed for the diffusion of the reagent, the effects were studied under a microscope. When a few drops of concentrated ammonia are added, the color reaction appears immediately. The characteristic feature of the reaction is the development of a sharp pink color in the regions susceptible to the test. As soon as the reaction ceases, the color rapidly fades away. In general, the color reaction appears first at the region of highest concentration, and, as it progresses to regions of lower concentration, the color becomes lighter and lighter. Since the intensity of the color depends upon the concentration of the -SH group in the tissues, it can be taken as a measure of the relative regional concentration of the -SH group. According to Giroud and Bulliard (see Serra, 1946), the red coloration can be stabilized by a pretreatment with zinc acetate instead of acetic acid. This procedure, however, was not found so successful in our material as Coldwater's modification.

The nitroprusside reaction detected in *S. fossularis* may be completely inhibited by treatment with formaldehyde. This seems to suggest the presence of the compounds of glutathione, cysteine, and methionine types. But, according to the opinion of Giroud (1931), which was also accepted by Coldwater (1933), the reaction may be caused by the protein-fixed sulphydryl rather than by glutathione in the cells.

RESULTS

Upon even a casual inspection of the nitroprusside reaction test, it becomes immediately apparent that in *S. fossularis* the distribution of -SH in the different body regions is not at all uniform. In a normal worm without fission, the coloration of the reaction appears first in the head region and then extends posteriorly with decreasing intensity of the pink color. Almost simultaneously or a short time afterward, the tail end of the worm also starts to react, and the intensity of the color decreases anteriorly. As shown in Figure 1, the arrows indicate the direction of decrease of the coloration, and the black dots represent the intensity of the pink color. It is clear that in a worm without fission there are two opposed gradients of the nitroprusside reaction, one extending from the head region posteriorly and the other from the anal region anteriorly. The former appears to be stronger, because it reacts at a greater speed than does the latter. The whole digestive tract has a higher content of the -SH group than do adjacent regions. Figures 1 and 2, *a* and *b*, also show that the mouth region and the pharnyx at the anterior, and the anal region at the posterior, ends give the deepest color reaction.

In the worms undergoing fission the pattern of the nitroprusside reaction appears to be somewhat modified by the development of the fission zone, which shows a striking color reaction. The intensity of the color developed in the fission zone seems to depend upon the stage of the zone development, that is, the earlier stages are not so strongly reactive as are the later ones. On the two sides of the fission zone the reaction appears to form two gradients extending toward the anterior and posterior ends of the worm, respectively. Figure 3 shows the patterns of the reaction of such a worm with two

fission zones. In the more advanced fission zone (Fig. 2*c*), the color reaction in the regions that will soon differentiate into mouth and pharnyx on one side of the fission plane and into anus on the other will, in due time, show an even stronger reaction, which will also appear in the new individual following complete separation. In the case of the worm that develops a chain of fission zones, the zoöids derived from a single segment, as described previously (Chu, 1945), show a gradient pattern of the nitroprusside reaction independent of the parent worm (Fig. 4).

In a starved worm the nitroprusside reaction generally indicates a lower concentration of the -SH group. The amount of the decrease of -SH appears to be in direct proportion to the duration of starvation. In a starved worm with fission zone, however, if the fission zone proceeds with its development, the -SH group revealed by the reaction will show a deeper color in the fission zone than in the adjacent regions. This is true even if starvation is prolonged. Nevertheless, the intensity of the color appears to be less than in the corresponding regions of a nonstarved worm with fission zone. If the fission zone ceases to differentiate after prolonged starvation, it will soon undergo regression, and the region will no longer show any sign of greater amount of the -SH group than adjacent regions. Such fission zones are no longer visible (Chu, 1946).

The pieces that are newly cut give the same color reaction as do the corresponding parts in an intact worm (Fig. 5, *a*). If the pieces are allowed to regenerate for about 2 days, swelling occurs at both ends as a result of cell proliferation, and the wound heals. These regenerating regions react more rapidly than other parts (Fig. 5, *b*). If the test is applied to a 3-day-old regenerating piece, an even more intense reaction will result, especially at the extreme tips of both ends. This indicates the increase of -SH concentration with the progress of regeneration.

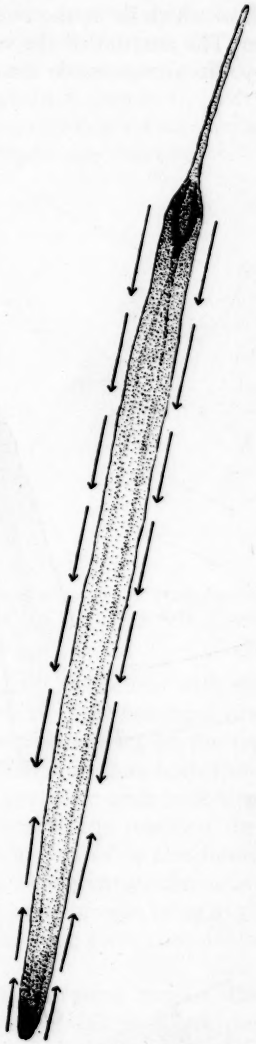

FIG. 1.—Schematic diagram showing the gradient color pattern of the nitroprusside reaction in a normal *S. fossularis* without fission. The appendages of the worm are not shown. The arrows indicate the direction of progress of the color reaction, and the black dots, the intensity of the color.

DISCUSSION

The data presented in this paper have clearly shown that, in the worm *S. fossularis*, the -SH group content is distributed in two gradient patterns, the high ends of which lie at the two ends of the worm. The amount of the substance shown by the nitroprusside reaction becomes progressively less toward the middle region of the body. The digestive tract also has a higher content of the -SH group, probably on account of the sulphydryl compounds contained in the food. In this connection it is of interest to recall the work on *Tubifex* by Coldwater (1933), who demonstrated that, when the

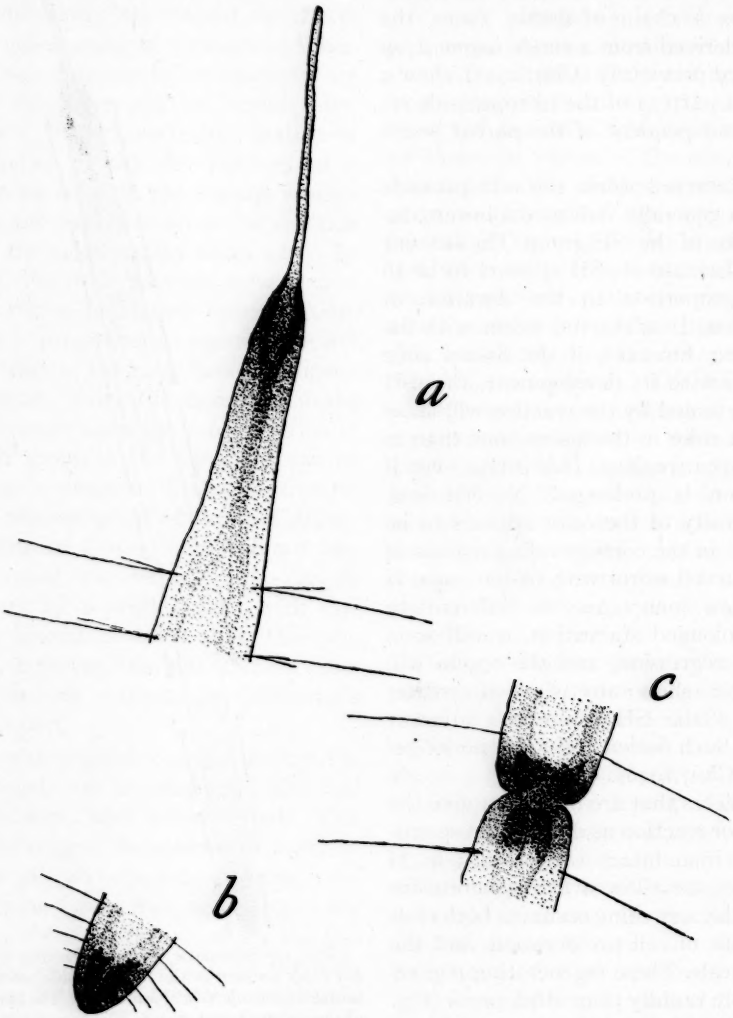

FIG. 2.—Regions showing deep coloration in nitroprusside reaction. *a*, the mouth part; *b*, the fission zone; *c*, the tail bud.

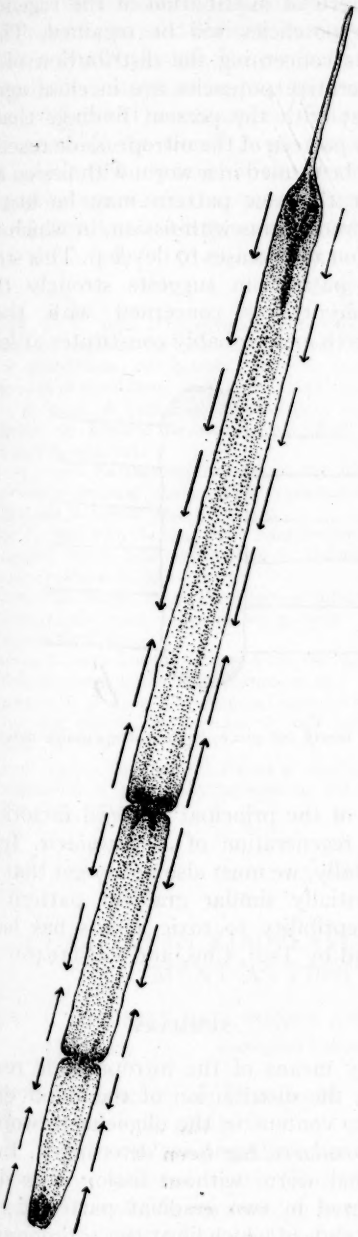

FIG. 3.—Gradient pattern of a worm with fission. Explanation in text.

organism had been kept in solutions containing sulphydryl compounds, the most intense reaction showed up in the gut epithelium of the posterior part of the body. He explained the phenomenon as caused by the respiratory function of the posterior end of the gut, the endoderm serving as an absorption surface for the sulphydryl. In *S. fossularis*, however, the pharyngeal region gives an even more intense nitroprusside reaction.

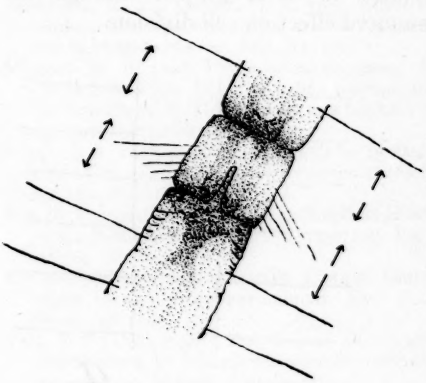

FIG. 4.—Color pattern in nitroprusside reaction in the region of a chain of zoöids. Explanation in text.

The results of the test with the regenerating pieces of *S. fossularis* are similar to Coldwater's finding on the regenerating planarians, in that both the anterior and the posterior ends give a more intense nitroprusside reaction. In experiments with *Tubifex* he also found a similar result. Apparently, the actively proliferating mass of cells lying at the tip of the regenerating piece contains more sulphydryl.

The regenerating tissues, the fission zone, and the tail bud are potentially capable of unlimited growth. The cells in these tissues are evidently more active, hence possessing a higher metabolic function similar to the embryonic tissues. That these tissues are rich in sulphydryl

suggests the possibility that sulphydryl has something to do with the increase of metabolic activity. As maintained by many biochemists and physiologists, glutathione, which contains the sulphydryl group, plays an important role in the enzyme system for respiration. It is possible that the increase of enzymatic activity may be a function of the -SH group contained in glutathione. This may explain why the -SH group is intimately related to tissue growth through pronounced effect on cell division.

pattern of distribution of the regenerative potencies will be regained. These facts concerning the distribution of regenerative potencies are in close agreement with the present findings that a new pattern of the nitroprusside reaction can be formed in a worm with fission and that the new pattern may be lost in starved worms with fission, in which the fission zone ceases to develop. This striking parallelism suggests strongly that sulphydryl is concerned with tissue growth and probably constitutes at least

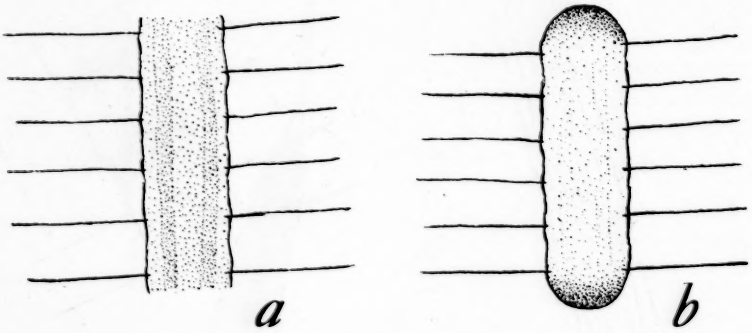

FIG. 5.—*a*, The nitroprusside reaction color pattern of a newly cut piece; *b*, the nitroprusside reaction color pattern of a 2-day-old regenerating piece.

The directly visible gradient patterns of the nitroprusside reaction in *S. fossularis* are of special interest in connection with our earlier attempt to explain the regeneration phenomena of the worm by the distribution of regeneration potencies. In a previous communication we postulated two opposed, but overlapping, concentration gradients of regenerative potencies, beginning from the anterior and posterior ends, respectively. In the worm with fission, these two gradients are redistributed in accordance with what will be determined by development of the fission zone (Chu, 1946, Fig. 7, p. 236). In case the fission zone disappears as a result of cutting or of starvation, the gradients will disappear, and the original

one of the principal material factors in the regeneration of *S. fossularis*. Incidentally, we must also not forget that an essentially similar gradient pattern of susceptibility to toxic agents has been found by Tsui, Chu, and Pai (1946).

SUMMARY

By means of the nitroprusside reaction, the distribution of the sulphydryl group content in the oligochaete worm, *S. fossularis*, has been determined. In a normal worm without fission it is distributed in two gradient patterns, the high ends of which lie at the anterior and posterior ends, respectively. The digestive tract also shows a higher concentra-

tion of the substance, especially in the pharyngeal and anal regions. In the case of a worm with fission, the two adjacent sides of the fission zone appear to develop new gradients. These disappear following starvation of the worm. A piece that is cut from any body level of the worm reveals the same reaction as that region would show in an intact worm. If it is allowed to regenerate for 2 or 3 days, the regenerating tissue will develop a new gradient pattern, decreasing from the cut end or ends, the intensity of the color reaction depending on the amount of tissues regenerated. A possible relationship between the sulphydryl gradient and the regeneration potency gradient is discussed.

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CHANGES IN RIBOFLAVIN DURING EMBRYONIC DEVELOPMENT AS FUNCTIONS OF THE EMBRYO[1]

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THE presence of fluorescent compounds such as riboflavin, pterines, etc., in various organisms has been repeatedly pointed out (Rosenberg, 1942). Much of this information has been obtained from studies on the adult or fully formed animal, while little data for embryonic forms seem available. Pterines have long been associated with problems of pigment formation in insects, and much of our knowledge of the chemistry and other properties of these compounds has been derived from such investigations (Becker, 1936; Hopkins, 1941;

[1] Aided by a grant from the National Institute of Health.

Busnel and Drilhon, 1942). Recently it has been shown that during the embryonic development of the grasshopper pterines are probably derived from the riboflavin of the egg (Bodine and Fitzgerald, 1947*a*, *b*). As a matter of fact, the entire riboflavin content of the egg appears to be converted quantitatively into pterines during the course of the animal's development. Since the egg of the grasshopper is a cleidoic or closed system, it serves well as experimental material in following through the various changes in riboflavin during the course of embryonic development. As previously pointed out (Bodine and Allen, 1941), it is possible to destroy completely the embryo of the egg by the use of X-irradiation and to leave intact the extra-embryonic structures, such as serosa, yolk, etc. The present paper deals with results of experiments in which the embryo was destroyed by X-irradiation very early in development and the subsequent changes in riboflavin of the egg were noted. The extent to which these changes are due to the embryo per se, as well as to extra-embryonic structures, such as the serosa, has been specifically investigated.

MATERIALS AND METHODS

Materials and methods employed were essentially similar to those recently described (Bodine and Fitzgerald, 1947*a*, *b*). Young eggs (5–7 days) of the grasshopper (*Melanoplus differentialis*) in lots of 1,000–5,000 were carefully prepared and subjected to X-rays to destroy the contained embryos. Control lots were treated in precisely the same manner without exposure to X-rays. A dosage of 1,000 r was delivered to the eggs under the following conditions: 120 kv., 5 M.A., 30-cm. target distance, H.V.L. 0.15 cm. copper, 12.6 minutes.[2] Following treatment, the eggs were kept at 25° C. for 3 weeks, then transferred to 5° C. for 30 days, and finally returned to 25° C. and kept there until hatched. Samples of extracts of both X-rayed and control eggs were analyzed at regular intervals for riboflavin and other fluorescent compounds. Fluorophotometric and spectrophotometric determinations on egg extract as well as on dialyzed fractions were carried out at appropriate intervals throughout the eggs' development. Fluorometric readings are expressed in galvanomoter scale readings, 33.5 divisions equaling 0.1 γ riboflavin per cubic centimeter or per egg. Further details of methods are indicated below in the results of experiments.

RESULTS

WHOLE-EGG EXTRACT

Extracts of both X-rayed and control eggs were studied with the fluorophotometer at various periods throughout development. Inasmuch as all readings on different series are qualitatively alike, only results of typical experiments will be given in detail. In Table 1 are given values obtained at intervals for such a series from the day of X-irradiation to hatching. An inspection of these data shows no marked changes in fluorescence in the control or experimental eggs until late in postdiapause development. Most significant are the changes in the alkaline fluorescence of the control or nonirradiated eggs. Fluorescent compounds produced from the original riboflavin fluoresce at high pH values (pH 10) but are not reduced by sodium hydrosulphite at such pH values. That marked changes in the riboflavin of the control eggs occur is also evidenced by the fact that a marked pink coloration of the egg extract is noted in an alkaline medium. Upon separation and purification of these

[2] Acknowledgment is gratefully made to Dr. H. Dabney Kerr, of the Department of Radiology, for carrying out the irradiation.

compounds, definite crystals are formed, some of which are distinctly pink in color. A comparison of the control or nonirradiated eggs with the X-irradiated ones shows rather clearly a marked lack of high fluorescent values for the irradiated eggs for the alkaline range. Apparently, the presence of an embryo seems essential for the conversion of the riboflavin into the alkaline fluorescing compounds (pterines). From previous experiments (Bodine and Allen, 1941) it is evident that, in eggs subjected to X-ray, only the serosa and yolk cells remain. These highly resistant extra-embryonic cells are not killed, inasmuch as the respiration of such eggs follows a regular curve throughout the remainder of their devel-

TABLE 1

GALVANOMETER SCALE READINGS OF SPECTROPHOTOMETER FOR WHOLE-EGG EXTRACTS

A scale reading of 33.5 equals 0.1γ riboflavin per cubic centimeter or per egg. Sodium hydrosulphite crystals added to extract to reduce riboflavin. All eggs from same lot.

	Fluorometric Readings	
	Acid (pH 4.5)	Alkaline (pH 10)
	Original Extract	
Control	Extract = 28.0; +crys. = 7.5 → 21.5	Extract = 8.0; +crys. = 9.0
X-rayed	Extract = 28.0; +crys. = 7.0 → 21.0	Extract = 8.0; +crys. = 8.5
	38 Days at 25° C	
Control	Extract = 28.5; +crys. = 10.5 → 18.0	Extract = 7.5; +crys. = 9.5
X-rayed	Extract = 28.5; +crys. = 10.5 → 18.0	Extract = 7.0; +crys. = 8.0
	35 Days at 5° C.	
Control	Extract = 32.0; +crys. = 11.0 → 21.0	Extract = 9.0; +crys. = 10.0
X-rayed	Extract = 30.0; +crys. = 10.0 → 20.0	Extract = 8.0; +crys. = 9.0
	19 Days at 25° C.	
Control	Extract = 36.0; +crys. = 9.0 → 27.0	Extract = 44.0; +crys. = 47.0 pink
X-rayed	Extract = 32.0; +crys. = 9.5 → 22.5	Extract = 9.5; +crys. = 12.0 pinkish?

TABLE 2

GALVANOMETER SCALE READINGS OF SPECTROPHOTOMETER FOR DIALYZED EGG EXTRACTS

A scale reading of 33.5 equals 0.1γ riboflavin per 1.25 egg. "Inside" refers to inside of shell, while "outside" refers to dialyzate.

	FLUOROMETRIC READINGS	
	Acid (pH 4.5)	Alkaline (pH 10.)
	38 Days at 25° C.	
Controls	Inside: extract = 10.0, +crys. = 7.5 — 2.5	Extract = 4.5, +crys. = 5.5
	Outside: extract = 13.5, +crys. = 2.0 — 11.5	Extract = 3.0, +crys. = 3.5
X-rayed	Inside: extract = 11.0, +crys. = 7.0 — 4.5	Extract = 4.5, +crys. = 5.0
	Outside: extract = 12.0, +crys. = 2.0 — 10.0	Extract = 3.0, +crys. = 3.5
	35 Days at 5° C.	
Controls	Inside: extract = 12.0, +crys. = 9.0 — 3.0	Extract = 6.0, +crys. = 6.0
	Outside: extract = 10.0, +crys. = 2.0 — 8.0	Extract = 4.0, +crys. = 4.0
X-rayed	Inside: extract = 15.0, +crys. = 9.0 — 6.0	Extract = 7.0, +crys. = 8.0
	Outside: extract = 10.0, +crys. = 2.0 — 8.0	Extract = 3.0, +crys. = 3.5
	19 Days at 25° C.	
Controls	Inside: extract = 14.0, +crys. = 9.5 — 4.5	Extract = 9.0, +crys. = 10.0
	Outside: extract = 18.5, +crys. = 2.5 — 16.0	Extract = 16.0, +crys. = *16.0*
X-rayed	Inside: extract = 11.0, +crys. = 8.0 — 3.0	Extract = 6.0, +crys. = 6.0
	Outside: extract = 10.5, +crys. = 2.5 — 8.0	Extract = 4.5, +crys. = 4.5

opment. The earlier in postdiapause development that determinations are carried out, the smaller is the amount of riboflavin found converted into alkaline fluorescing compounds. That several types of alkaline fluorescing compounds are eventually formed is demonstrated by the fact that at least three have been obtained in crystalline form. Their physicochemical nature will be described at some future date.

DIALYSIS EXPERIMENT

Riboflavin and its derivatives pass through ordinary sausage-casing dialyzing shells, and this property makes possible further analysis of the fluorescent compounds of the whole-egg extracts. Separation of riboflavin and its derivatives from other inherent fluorescent compounds thus becomes a relatively easy way of following through changes in the one compound without the disturbing effects of those already present in the egg (Bodine and Fitzgerald, 1947*a*). In Table 2 are given fluorometric values of dialyzing experiments for both X-rayed and control eggs. An examination of these data again shows no marked changes in the fluorescence of the two types of eggs until late in postdiapause development. Throughout the development of the eggs the dialyzable and nondialyzable fluorescent fractions show a rather striking constancy for all eggs examined. Most significant are the marked changes in the control or nonirradiated eggs during postdiapause development. A marked increase in the dialyzable fluorescent fractions at both low and high pH values is clearly indicated. In striking contrast to this are the constant low values for the X-rayed or experimental series of eggs. During the entire period of the experiment the fluorescence of the irradiated or nonembryo-containing eggs remains practically constant, indicating that without the embryo little if any change in the riboflavin takes place.

SPECTROPHOTOMETRIC DETERMINATIONS

As a further check on changes in the riboflavin content of the eggs, spectrophotometric studies have been carried out on the transmittance of the extracts at different wave lengths. As indicated in previous publications (Bodine and Fitzgerald, 1947*a*, *b*), such results seem of value only in conjunction with those for the fluorescence of the same extracts. Typical spectral transmittance curves are shown in Figure 1. Transmittance curves for whole-egg extracts are of significance only in indicating marked changes in the physicochemical nature of the egg extract with development of the embryo, and these are probably correlated with changes in the amount and character of the proteins which are made into the embryo proper. Of especial importance, however, are the transmittance curves for the dialyzable fractions of the egg extracts. Dialyzed fractions of young eggs show transmittance curves similar to those for pure riboflavin (Fig. 1). As changes in the riboflavin occur late in postdiapause, corresponding changes in the transmittance curves are also noted (Fig. 1). In comparing these changes in the irradiated and control eggs, it can be seen that most marked are those occurring in the nonirradiated or embryo-containing egg, indicating that the presence of an embryo seems essential for changes in the contained riboflavin.

No changes in the nonreducible, nondiffusible fluorescent fractions of either normal or X-ray–treated eggs have been found.

DISCUSSION

In previous communications it was pointed out that changes in riboflavin are perhaps those concerned with the

production in insects of the so-called "pigment-forming compounds," pterines (Bodine and Fitzgerald, 1947*a*, *b*). As a result of further study of these substances, it can be definitely stated that in the grasshopper at least three separate and distinct pterines are thus produced. The chemical and physicochemical na-

embryonic significance of riboflavin exist, and all seem to indicate its importance for the normal metabolic processes of the organism. During the development of the grasshopper egg the significance of riboflavin for the early developmental periods is obscure. From the present data one may assume that this compound plays

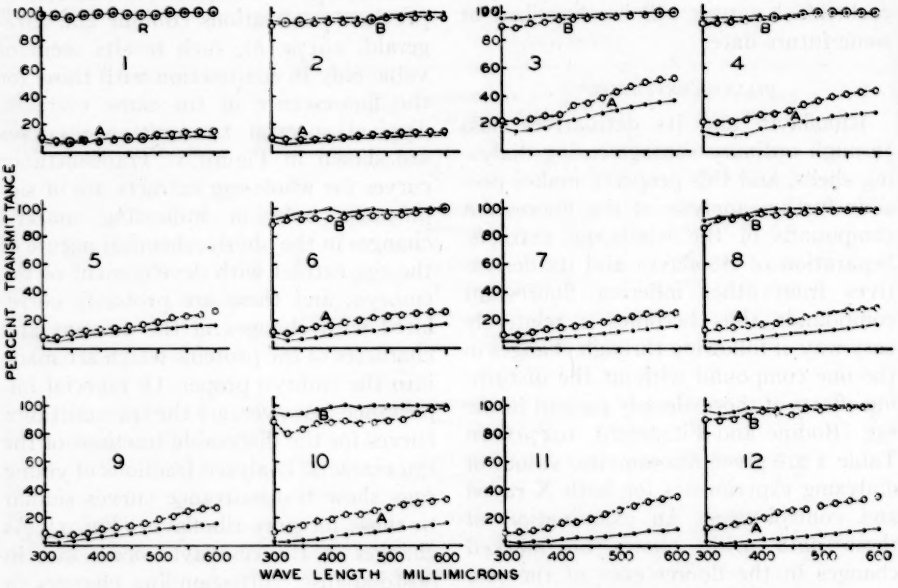

FIG. 1.—Shows transmittance of control and X-rayed whole-egg extracts and dialyzed fractions at different wave lengths. *Ordinate*, per cent transmittance. *Abscissa*, wave lengths in millimicrons. Solid circles for pH 4.5, open circles for pH 10. *A* = whole-egg extracts (inside). *B* = dialyzed fraction (outside). *R* = pure riboflavin (0.1 γ/cc). *1* = beginning of experiment (6/3/47) before dialysis. *2* = same as 1 except dialyzed. *3* = control, nonirradiated, dialyzed (6/23/47). *4* = same as 3 except for X-rayed eggs. *5* = control, nondialyzed (7/23/47). *6* = same as 5, except dialyzed (7/23/47). *7* = same as 5, except for X-rayed eggs (7/23/47). *8* = same as 7, except dialyzed (7/23/47). *9* = control, nondialyzed (8/11/47). *10* = same as 9, except dialyzed (8/11/47). *11* = same as 9, except for X-rayed eggs (8/11/47). *12* = same as 11, except dialyzed (8/4/47).

ture of these will be discussed at a later date. The significance of the conversion of riboflavin by the embryo per se into pterines is of considerable interest in view of the scanty and almost negligible amount of information concerning the role of such compounds during the embryonic development of an organism. Large amounts of data for the post-

little part in the developmental process until rather late and then appears destined to be transformed into pterines, which are later laid down as pigments in various parts of the adult organism (Hopkins, 1941). Curiously, the amounts of pterines found seem conditioned by the amount of original riboflavin present in the egg. This point, however, needs

further elucidation. Values for riboflavin originally present in the egg, however, approach those for the crystalline pterines found at the end of the developmental periods. That the fluorescence of the pterines produced is due to the fluorescent fraction of the riboflavin molecule seems logical if the structural formulas of the two compounds are compared (see Fig. 2).

Another point of considerable theoretical interest at the present time is the nonembryo-containing eggs have been noted. Extra embryonic cells, such as those of the serosa, which are not susceptible to doses of X-rays as high as 1,000 r, are apparently not able appreciably to change riboflavin into pterines. It thus seems reasonable to suggest that such powers of conversion are the properties of embryonic cells and especially of those readily susceptible to X-ray. That marked changes or differences in the metabolism or use of riboflavin by dif-

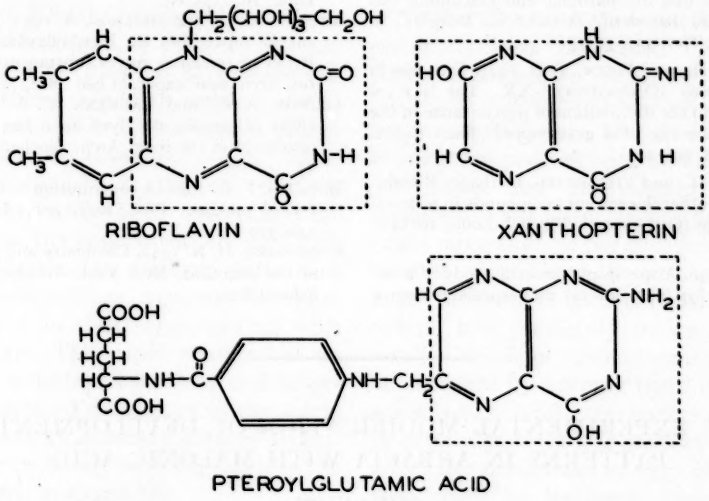

FIG. 2

possible relation that the pterines may have in contributing to the formation of folic acid. The structural formulas in Figure 2 suggest such a relationship. Data on this point for vertebrate embryos should prove of considerable value in view of the importance of folic acid for the normal functioning of the adult organism.

That the conversion of riboflavin into pterines in the grasshopper egg is a function of the embryo per se seems rather clearly established. No marked changes in the riboflavin content of irradiated or ferent types of cells occur has been demonstrated by the work of Griffin and Baumann (1946) on liver cells treated with carcinogenic reagents. Liver cells thus treated show marked reductions in the amount of riboflavin utilized, when compared with the normal highly differentiated cells.

CONCLUSIONS

1. A study has been made on the effects of X-irradiation on the egg of the grasshopper, *M. differentialis*, in order to determine the part played by the em-

bryo in the changes in riboflavin of the egg.

2. Fluorometric and spectrometric data are presented for both normal and X-irradiated eggs.

3. Eggs in which the embryo is destroyed by X-irradiation show little if any change in their riboflavin content. Normal or embryo-containing eggs show marked changes in riboflavin, especially late in embryonic development.

4. Riboflavin is converted into pterines only in nonirradiated or normal eggs.

5. The conversion of riboflavin into pterines seems to be a specific property of the embryo per se and not of differentiated extra-embryonic structures, such as the serosa.

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THE EXPERIMENTAL MODIFICATION OF DEVELOPMENTAL PATTERNS IN ARBACIA WITH MALONIC ACID

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THE experimental modification of developmental patterns in echinoderms may be accomplished by a variety of agents.[2] Most modifications are the results of inhibition or stimulation of some sort during critical periods. Certain regions of the developing embryo are more susceptible to inhibiting or stimulating agents than other regions are; and this differential susceptibility may lead to changes in proportion and form as the animal continues its development. An initial differential inhibition of a region may be followed by differential recovery when the larva is returned to a normal environment, and this may produce modification in the opposite direction. If the agent used is only slightly

[1] This work was undertaken while the author was still a member of the biology department of Wayne University. Laboratory fees and certain other expenses were carried by this institution. For these and many other considerations the writer is most grateful.

[2] See Child (1941) for the most recent review of literature dealing with the modification and control of developmental patterns in echinoderms.

inhibitory, certain regions may become conditioned or acclimated to it more readily than do other regions and by this means also alter the course of development.

The over-all general effects of various inhibitory agents may be quite similar, but there are some differences. Lithium chloride and sodium thiocyanate are both inhibitory and, when applied to the developing echinoderm egg, tend to produce entodermization if the concentrations and exposure periods are properly controlled (Child, 1936; Rulon, 1941). On the other hand, if these agents are used before fertilization in Ca-free sea water, lithium tends to produce entodermization, while thiocyanate causes *ectodermization* (Lindahl, 1933, 1936; Rulon, 1946). It is thought (Rulon, 1946) that, while both agents are inhibitory in the concentrations used and tend to flatten the gradient pattern of the unfertilized egg, recovery is much more rapid in those eggs treated with thiocyanate. This rapid recovery of an egg with a flattened gradient and subsequent decreased dominance of the apical end leads to an increased scale of organization, with ectoderm being produced at the expense of entoderm.

The following experiments on the eggs of *Arbacia* were attempts to probe further into the mechanisms of developmental patterns by the use of malonic acid, an agent which is reputed to inhibit markedly the action of the enzyme succinic dehydrogenase.

MATERIAL AND METHODS

This study was made during the summer of 1947 at Woods Hole. The eggs of *Arbacia punctulata* were obtained in the following standard manner: Freshly collected urchins and scissors were washed in tap water, after which an incision was made around the peristome and Aristotle's lantern removed. The animals were then drained of their body fluid and placed, aboral side down, in finger bowls. Only the eggs from females that shed abundantly were used. The eggs from a single female were washed several times in sea water before proceeding with the experiments. Test and control eggs were always from the same batch. Both virgin and fertilized eggs were kept in finger bowls on the water table throughout the course of the experiments. Test solutions were always isotonic with sea water.

At the time that these particular experiments were performed (June and July), considerable difficulty was encountered in obtaining batches of 90–100 per cent fertile eggs. Despite every precaution to prevent contamination by perivisceral fluid and despite repeated washings in sea water, it was impossible to get more than 50 per cent fertilization in most cases. Since the author (Rulon, 1946) and others (Schechter, 1937; and others) had previously shown that the fertilizable life of echinoderm eggs could be extended by keeping them in low-Ca sea water, several experiments were run to determine if Ca-free sea water[3] would affect the percentage of eggs in a batch that could be fertilized. Several tests showed that eggs giving 25–50 per cent fertilization could be stimulated to 90–100 per cent fertilization by allowing the freshly shed and washed eggs to stand in Ca-free sea water for 8 hours before returning to sea water and adding a few drops of sperm suspension. The development of eggs treated before fertilization with Ca-free sea water, as well as the resulting plutei, was completely normal.

The experiments here described were with eggs that had undergone a prefertilization treatment with Ca-free sea water, although they served only to con-

[3] Distilled water, 10,571 cc.; NaCl, 307 gm.; KCl, 8 gm.; $MgSO_4$, 66 gm.; $NaHCO_3$, 2 gm.

firm certain effects of malonic acid that had been found in other experiments on inferior eggs.

EXPERIMENTAL

1. THE EFFECT OF MALONIC ACID WHEN ADMINISTERED ONLY BEFORE FERTILIZATION

Virgin eggs were exposed to 100 cc. of Ca-free sea water plus 3.0 cc. of 0.036 M malonic acid for 6 hours, after which they were washed and fertilized in normal sea water. These eggs showed 95 per cent normal cleavage and developed into normal plutei at approximately the same rate as the controls (Ca-free sea water before fertilization). Twelve hours' exposure to this solution resulted in 95–100 per cent cleavage, but the cleavage pattern was often slightly abnormal. In spite of this effect the eggs usually developed into normal plutei. When the treatment was continued for 24 hours, normal plutei still developed from a high percentage of the eggs. However, a sizable number failed to develop past the late blastula stage and were to be found at the bottom of the culture dishes. These inhibited bottom forms often had cells protruding from one area, and occasionally two were fused together.

When unfertilized eggs were treated for 12 hours with 100 cc. of Ca-free sea water plus 4.0 cc. of 0.036 M malonic acid, the effects were more pronounced. Only 30–40 per cent cleaved, and observations 3 hours after fertilization showed the developing eggs to be in 2-, 3-, 4-, 8-, and 12-cell stages. Cleavage furrows were usually irregular, with the boundaries of the blastomeres often hard to see. Cells were commonly of unequal size. The controls at this time were usually in the 16-cell stage. A few of the test eggs developed into plutei that were normal except for slightly inhibited oral lobes. Most of the cleaving eggs, however, developed no further than slow-moving, cell-filled, irregular, bottom blastulae.

Six hours' exposure of unfertilized eggs to 100 cc. of Ca-free sea water plus 5.0 cc. of 0.036 M malonic acid was drastic. Less than 1 per cent of the eggs cleaved following fertilization. The eggs appeared darkened, with a roughened cortex and a tendency to clump. Observations showed some of the eggs exuding clear droplets from their surfaces.

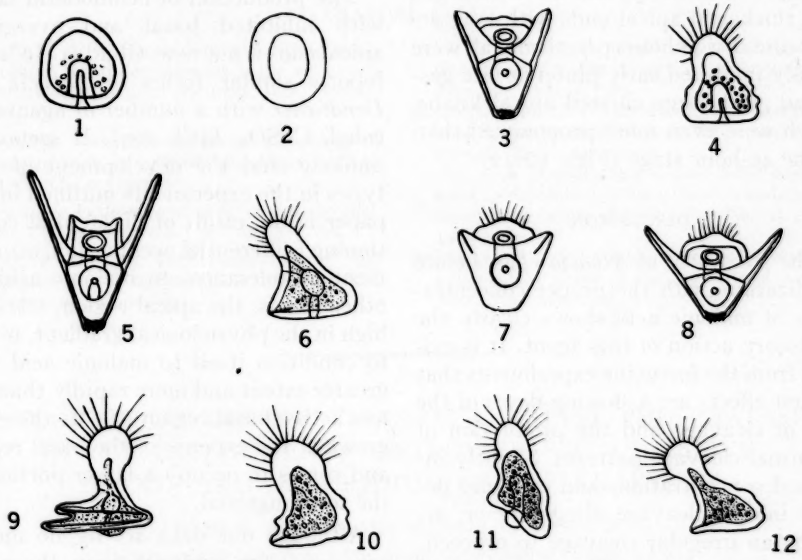
2. THE EFFECT OF MALONIC ACID WHEN ADMINISTERED AFTER FERTILIZATION

When eggs in the 1- and 2-cell stages were placed in 100 cc. of sea water plus 35 cc. of 0.0036 M malonic acid, there was a slight retardation in cleavage rate. After 13 hours, when the controls were actively swimming bilateral gastrulae (Fig. 1), the treated eggs showed no gastrulation but were free-swimming spherical, or slightly elongate, blastulae, with no flattening of the ventral surface (Fig. 2) or bottom; slow-moving, spherical blastulae, with some still in their fertilization membranes. After approximately 24 hours, when the controls had developed into young plutei (Fig. 3), the experimental lots were usually gastrulae, which were wrinkled around the basal regions but with thickened apical ends, showing long active cilia (Fig. 4). By 40 hours the controls had developed into normal plutei, with long anal arms and fairly well-developed oral lobes (Fig. 5). The experimental forms, on the other hand, were inhibited, cell-filled plutei, with the oral lobes often extended and thickened to form active ciliated apical knobs (Fig. 6).

Embryos, developing in 100 cc. of sea water plus 35 cc. of 0.0036 M malonic acid, were returned to normal sea water after 13 hours. At 24 hours, when the controls had developed into young plutei (Fig. 3), the treated individuals were

still quite similar to those that had been in the malonic acid solution for the full experimental period. Twelve hours later (36-hour stage) these forms, although still darkened and inhibited as a whole, showed considerable recovery. Short anal arms had developed, while the oral lobe was usually slightly enlarged, with an active ciliated tip (Fig. 7). At the 48-hour stage plutei with fairly long anal arms had usually developed. In these the angles of the anal arms were often increased and the oral lobes widened but not well differentiated (Fig. 8). A number with aberrancies, such as extra anal arms, were noted. When the developing eggs were left in the test solution for the first 24 hours and the larvae (Fig. 4) were then returned to normal sea water, the pattern was quite different. At 48 hours almost all were free-swimming, with large, pigmented, ciliated, apical lobes. Anal arms were decreased in size or absent. When they did appear, the angles of the skeletal rods were often reduced to such an extent that they were almost parallel (Fig. 9).

Eggs that had developed to the late-cleavage stages (approximately 5 hours) in normal sea water were placed in a solution of 100 cc. of sea water plus 35 cc. of 0.0036 M malonic acid for the remainder of the experimental period. After 10 hours' exposure to the malonic acid, when the controls were actively swimming mid-gastrulae, the test embryos were still spherical thick-walled blastulae on the bottom of the culture dishes. Some showed slight ciliary movement, and most were still in their fertilization membranes. At the 24-hour stage, with the controls young plutei



FIGS. 1–12.—Fig. 1, 13-hour control larva. Fig. 2, 13-hour malonic acid–treated larva. Fig. 3, 24-hour control larva. Fig. 4, 24-hour malonic acid–treated larva. Fig. 5, 40-hour control larva. Fig. 6, 40-hour malonic acid–treated larva. Fig. 7, 36-hour larva, treated with malonic acid first 13 hours. Fig. 8, 48-hour larva, treated with malonic acid first 13 hours. Fig. 9, 48-hour larva, treated with malonic acid first 24 hours. Figs. 10–12, 48-hour larvae treated with malonic acid, starting at approximately 5 hours after fertilization.

(Fig. 3), those that had been treated were still chiefly thick-walled blastulae, but with slight invaginations beginning to show in some. These forms were even more retarded than those that had been in the test solution the whole of the developmental period. By the 40-hour stage they had developed chiefly into cell-filled gastrulae, with wrinkled, inhibited basal regions, only slight evidence of bilaterality, and much enlarged and thickened apical ends with long active cilia. By 48 hours practically all were grossly inhibited early plutei or late gastrulae, with large ciliated apical knobs, which were even more pronounced than at the 40-hour stage (Figs. 10–12).

DISCUSSION

The treatment of *Arbacia* eggs, before fertilization, with the proper concentrations of malonic acid shows clearly the inhibitory action of this agent. It is evident from the foregoing experiments that its first effects are a slowing-down of the rate of cleavage and the production of abnormal cleavage patterns. Slightly increased concentrations and exposure periods inhibit cleavage altogether or, allowing an irregular cleavage to proceed, prevent gastrulation. These data are of interest, in that certain activities or substances in the still unfertilized egg can be so affected that future potentialities may not be realized. Treatment of the virgin egg to such an extent that gastrulation is inhibited, even though cleavage proceeds, may offer certain clues to the dynamics of development.

Treatment of fertilized eggs with malonic acid inhibits both rate of cleavage and gastrulation. Under the conditions of our experiments it does not produce exogastrulation. Instead, inhibited gastrulae or early plutei, with wrinkled basal regions and enlarged apical knobs, appear following continuous exposure (Figs. 4 and 6). This condition is even more pronounced if the late-cleavage stages are placed in the malonic acid solution (Figs. 10–12). The effects are less drastic, and considerable differential recovery (increased angles to anal arms, etc.) takes place when eggs are exposed to the agent only during the first hours of the developmental period (Figs. 7–8).

The production of echinoderm larvae with inhibited basal and overgrown apical ends is not new. Child (1916, 1936) reports similar forms in *Arbacia* and *Dendraster* with a number of agents (alcohol, $CuSO_4$, LiCl, etc.). It seems not unlikely that the development of such types in the experiments outlined in this paper is the result of differential conditioning (differential acclimation, acquirement of tolerance) to malonic acid. In other words, the apical region, standing high in the physiological gradient, is able to condition itself to malonic acid to a greater extent and more rapidly than the less active basal region can. It therefore grows at the expense of the basal region and comes to occupy a larger portion of the total material.

Although our data are by no means complete, the evidence from these experiments may indicate a gradient of succinic dehydrogenase activity which corresponds with the physiological gradient pattern (see Child, 1941) that has long been known to exist in the echinoderm egg.

SUMMARY

1. When unfertilized eggs of *A. punctulata* were exposed for as much as 12 hours to 100 cc. of Ca-free sea water plus 4.0 cc. of 0.036 M malonic acid, cleavage rate was retarded, and cleavage pattern was usually highly irregular. Development usually went no further than slow-mov-

ing, cell-filled, irregular, bottom blastulae. Similar exposure to Ca-free sea water alone allowed normal development.

2. When fertilized eggs (1 and 2 cells) and 5-hour blastulae were exposed to 100 cc. of sea water plus 35 cc. of 0.0036 M malonic acid (adjusted to isotonic strength with sea water), cleavage and gastrulation were retarded. Development usually went on to give inhibited late gastrulae or early plutei with basal regions strongly inhibited, while the apical regions were much enlarged and extended to form highly active ciliated knobs.

3. Fertilized eggs exposed for only the first 13 hours of the developmental period to a solution of malonic acid showed considerable differential recovery on return to normal sea water.

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HISTOCHEMICAL OBSERVATIONS OF ALKALINE PHOSPHATASES IN THE INTEGUMENT, GASTROLITH SAC, DIGESTIVE GLAND, AND NEPHRIDIUM OF THE CRAYFISH[1]

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THE Crustacea are among the relatively few arthropods which continue to undergo periodic molting after attaining the adult form. In recent years interest has largely centered on the hormonal control of ecdysis in crustaceans. Among the many workers in this field, mention may be made of Kleinholz (1942) and Brown (1944), who have written reviews with extensive bibliographies; and Scudamore (1947), who has experimentally confirmed the theory that the sinus gland inhibits molting and associated changes in the crayfish. With the thought that there might be demonstrable histochemical changes in activity of alkaline phosphatases during the molting cycle, we have examined a number of organs of the crayfish.

[1] Aided by a grant from the Graduate Research Fund, University of Illinois.

MATERIALS AND METHODS

Fresh-water crayfish of the species *Cambarus virilis* were used in the investigation. The animals were kept in a large concrete tank, the floor of which was covered with gravel and crushed oyster shells. Running water, dechlorinated and aerated, at a temperature of 17° C. was

supplied to a depth of 3 inches. A 100-watt light bulb, suspended above the tank, provided illumination for a 15-hour period each day. The crayfish were given a varied diet of oatmeal, fish, earthworms, and canned, commercial dog foods.

Specimens sacrificed for study were adult males. Pertinent facts concerning them are tabulated in Table 1.

Gomori's (1941) technique for visualization of alkaline phosphatase as sulphides was employed. Pieces of the integument and digestive glands, entire gastrolith sacs (freed of gastroliths if present), and nephridia from the premolt, molting, and postmolt animals were fixed in ice-cold acetone, imbedded in paraffin, and sectioned at 10 μ. The sections were mounted so that each slide held two sections of each of the four organs from a crayfish representing a stage of the molting cycle. Five different incubating mixtures were made up with these substrates: yeast nucleic acid, adenylic acid, and fructose diphosphate from the Schwarz Laboratories, New York City; vegetable lecithin from the Pfanstiehl Chemical Company, Waukegan, Illinois; and sodium glycerophosphate (52 per cent alpha) from the Eastman Kodak Company, Rochester, New York. The incubating mixtures were buffered at pH 9. Slides with deparaffinized sections were incubated at 37° C. for 5, 24, and 72 hours. Eosin Y was used as a counterstain. Control sections were treated like the experimental ones, except that distilled water replaced the substrate in the incubating mixture.

TABLE 1

ALKALINE PHOSPHATASES IN THE CRAYFISH

Stage	Weight (Gm.)	Date	Remarks
Premolt	16.3	8/12/47	Old shell easily separated from underlying new one; gastroliths (2) weighed 50 mg.
Molting	16.5	8/15/47	In act of shedding old shell; gastroliths (2) weighed 130 mg.
Postmolt	39.6	7/10/47	Four days after molting; the new shell was hardened, and no gastroliths were evident

RESULTS AND DISCUSSION

INTEGUMENT

The integument of the crayfish consists of a dermis surmounted by a single cell-layer of chitogenous epithelium and a shell. The latter has a thin outer cuticle, which, according to Yonge (1932), is a secretion product of tegumental glands. Beneath the cuticle is the much thicker chitinous layer of the shell, which is lamellated and formed by the underlying epithelium.

Sections of the integument were taken from the mid-dorsal region of the cephalothorax, just anterior of its junction with the abdomen. In the premolt and molting animals the old shell was easily separated from the new one; and, since no calcification of the new shell had yet occurred, it was practicable to obtain sections of the integument which included the new shell. However, in the postmolt specimen the shell had hardened, and it was impossible to obtain complete paraffin sections. For that reason observations of the integument do not include the postmolt condition.

The nuclei of the chitogenous epithelium were found positive for alkaline

phosphatase at 5 hours' incubation in the premolt and molting animals with all substrates used. The cytoplasm of the epithelium displayed moderate amounts of the enzyme in both stages studied. Dephosphorylating activity, however, was noticeably stronger in the epithelium of the molting individual, especially in the distal ends of the cells (Fig. 1). According to the intensity of their dephosphorylation in the cytoplasm, the order of the substrates was: fructose diphosphate > glycerophosphate > adenylic acid > nucleic acid > lecithin.

Deep in the dermis of the premolt and molting animals, tegumental glands were observed. While the nuclei and cytoplasm of fully developed and degenerating glands were but faintly positive with all substrates, those of newly developing glands were strongly positive (Fig. 1). The latter are easily distinguished as aggregates of large nuclei with very little cytoplasm.

An interesting feature is the presence of phosphatase in the upper zone of the chitinous layer of the shell, just inside the cuticle (Fig. 2). Here the enzyme activity is much more intense in the premolt as compared with the molting specimen. In both specimens with adenylic acid and glycerophosphate as substrates dephosphorylation was evident at 5 hours' incubation, and with fructose diphosphate at 24 hours. No phosphatase reaction in the upper zone of the chitinous layer of the shell was obtained with the use of either lecithin or nucleic acid as substrates. It should be mentioned that control sections revealed no preformed phosphate or pigmentation in this zone.

The phosphatase localization in the upper portion of the newly formed chitinous layer is puzzling. The ducts of the tegumental glands open externally on the surface of the cuticle. All ducts observed were negative for the enzyme; and the cuticle, although appearing black in the photomicrograph because it is strongly eosinophilic, actually is free of brown or black sulphide. The site of the enzyme activity suggests dephosphorylation in one or more of the following processes: pigment formation, laying down of the cuticle, production of the glairy secretion found between the new and the old shell at exuviation, or salvage of materials from the old shell before molting.

In a section from the molting specimen (Fig. 3) incubated with lecithin as a substrate, a newly formed seta was observed. The sheath stained intensely with eosin, but the protoplasmic core was faintly positive for phosphatase.

GASTROLITH SACS

Herrick's (1895) description of the gastrolith sacs of the lobster applies equally well to the smaller crayfish. The two sacs are convex outpocketings, one of the right and the other of the left wall of the anterior chamber of the stomach. Laterally the cavity of each sac is lined with a single layer of columnar epithelium, which forms the lining of the stomach chamber. The cells of the sac are much longer than those of the rest of the epithelium. The free epithelial surface is covered by a cuticle. During the premolt period a gastrolith is formed in each sac and is lodged between the epithelium and the cuticle. At molting time the latter is shed, and the gastroliths are liberated into the lumen of the stomach. In our postmolt specimen, 4 days after ecdysis, we were unable to find gastroliths in the stomach. Presumably they had been broken up and dissolved by that time.

In the specimens examined, the epithelial nuclei of the sacs were positive for alkaline phosphatase, with all substrates employed, at 5 hours' incubation. According to intensity of enzyme activity

evoked in the cytoplasm, the substrate order was the same as in the integumentary epithelium.

In the premolt specimen there was a diffuse phosphatase stain in the cytoplasm of the epithelium, with, however, a much darker stain at the distal ends of the cells. In the latter sites the reaction must surely be related to deposition of the calcareous gastroliths.

There was no evidence that a new cuticle had yet been formed by the epithelium of the sacs in the molting crayfish (Fig. 4), and the cytoplasm displayed only a diffuse stain for the enzyme. The shorter epithelial cells at the margins of the sacs present a vacuolated appearance at molting. At this time they are engaged in producing a glairy secretion beneath the old cuticle, and their protoplasmic content stains heavily for alkaline phosphatase.

A newly developed cuticle, closely adherent to the epithelium of the sacs, was evident in the postmolt stage. In addition to a diffuse stain the epithelial cytoplasm showed a more intense reaction for the enzyme at the distal ends of the cells, where dephosphorylation appears to be related to production of the cuticle.

DIGESTIVE GLAND

The digestive gland of *C. virilis* is morphologically like that described by Jordan (1904) for the European crayfish, *Astacus fluviatilis*. The gland is composed of many blind tubules, or caeca, which communicate with the mid-gut. Each tubule is lined with a columnar epithelium furnished with a basement membrane and a distal striated border. The relatively thick caeca have narrow lumens.

Young cells, which are produced at the distal ends of the tubules and at intervals among matured cells along the sides, were strongly positive for alkaline phosphatase in both cytoplasm and nuclei with all the substrates at 5 hours' or more incubation (Fig. 5). Matured cells, however, were negative except in the molting crayfish, where the nuclei were strongly positive; while the cytoplasm, along with the basement membrane and striated border, were faintly so with all substrates, beginning at 5 hours' incubation (Fig. 6). Possibly the dephosphorylation activity at molting is related to release of stored food, such as glycogen.

NEPHRIDIUM

The paired excretory organs of *C. virilis* are like those of *A. fluviatilis*, described by Marchal (1892). Each organ consists of a glandular portion and a bladder. The former, often called the "green gland" because of its color, is a modified tubular structure, lined by a single layer of epithelium. The epithelial cells rest on a fine basement membrane. The green gland begins with a much partitioned and alveolated end-sac, or saccule, which discharges into the cortical tube, a wide, flattened tube with numerous saclike evaginations. This tube leads into the medullary or white tube, a long, convoluted tube of uneven diameter, whose squamous epithelial cells contrast with the larger cuboidal cells of the cortical tube. The medullary tube leads into the thin-walled bladder, which discharges externally by way of a short duct that opens on a tubercle of the first segment of the antenna.

Within the partitions and between the alveoli of the saccule is a thin layer of highly vascular connective tissue. A similar layer with a rich blood supply is found between the outpocketings of the cortical tube and between the convolutions of the medullary tube.

By cutting through the median longitudinal plane, sections were obtained which included the three principal divi-

sions of the green gland: saccule, cortical tube, and medullary tube. When tested for alkaline phosphatase with the five substrates, no qualitative difference was apparent according to whether the sections were from the premolt, molting, or postmolt animal.

The epithelial nuclei of the saccule, cortical tube, and medullary tube were positive with all substrates, except lecithin.

The epithelial cytoplasm of the saccule appeared faintly positive with fructose diphosphate as a substrate after 24 hours' incubation (Fig. 7). With the other four substrates the reaction was negative.

The cortical tube showed the most intense cytoplasmic, alkaline phosphatase activity of any part of the nephridium. All the substrates used except lecithin were dephosphorylated in the cytoplasm. The cuticuloid border of the epithelium displayed the strongest phosphatase reaction, followed next by the basement membrane and last by a diffuse stain throughout the cytoplasm (Figs. 8 and 9). Fructose diphosphate produced a somewhat stronger reaction than did adenylic acid, glycerophosphate, or nucleic acid. In regard to the substrates that yielded a positive reaction, we were unable to discern any qualitative predilection of cellular sites for a particular substrate.

The cytoplasm of the medullary tube was very faintly positive for alkaline phosphatase, except with the use of fructose diphosphate, which yielded a moderately positive reaction after 24 hours' incubation (Fig. 7).

The heavy concentration of alkaline phosphatase in the cortical tube of the crayfish nephridium leads to a comparison with the kidneys of vertebrate animals. Moog (1946) reviewed the literature on the distribution of renal phosphatase in vertebrates. Invariably in mammals, including man, and in other classes of vertebrates the phosphatase has been found most highly concentrated in the proximal convoluted tubules, while the distal tubules are negative. Moog points out that this distribution supports the hypothesis that alkaline phosphatase in the kidney is part of a glucose-resorbing cycle in which glucose molecules are removed from the tubular fluid by phosphorylation, and the phosphate-bearing molecules are then dephosphorylated by phosphatase and returned to the blood stream.

The crayfish nephridium, although far removed phylogenetically from the vertebrate kidney, roughly suggests a huge excretory unit of a vertebrate kidney; and it is interesting that its cortical tube, which would compare with the proximal tubule of the vertebrate organ, shows a similar intense activity of alkaline phosphatase.

SUMMARY

The dephosphorylating activities of alkaline phosphatases in the integument, gastrolith sac, digestive gland, and nephridium during stages of the molting cycle of the crayfish have been studied histochemically.

The organs studied, with the exception of the nephridium, display changes in phosphatase activity associated with the molting cycle.

The marked localization of alkaline phosphatase activity in the cortical tube of the crayfish nephridium compares with that reported by other investigators in the proximal convoluted tubules of the vertebrate kidney.

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PLATE I

B, blood
C, chitogenous epithelium
Cl, chitinous layer of shell
Ct, cortical tube
Cu, cuticle
E, epithelium of gastrolith sac
Es, ordinary epithelium of stomach
G, glairy secretion
M, medullary tube
S, saccule
T, young tegumental gland

FIG. 1.—Integument of molting crayfish. Incubated 72 hours with nucleic acid. ×330.

FIG. 2.—Integument of premolt crayfish. Incubated 72 hours with fructose diphosphate. ×330. The upper part of the chitinous layer of the shell is positive for alkaline phosphatase.

FIG. 3.—Integument of molting crayfish. Incubated 72 hours with lecithin. ×180. A developing seta is shown.

FIG. 4.—Gastrolith sac of molting crayfish. Incubated 72 hours with nucleic acid. ×180.

FIG. 5.—Digestive gland of molting crayfish. Incubated 72 hours with nucleic acid. ×330. The distal end of a tubule is cut lengthwise, and its strong positive stain for alkaline phosphatase contrasts with the faintly positive reaction of the neighboring matured cells.

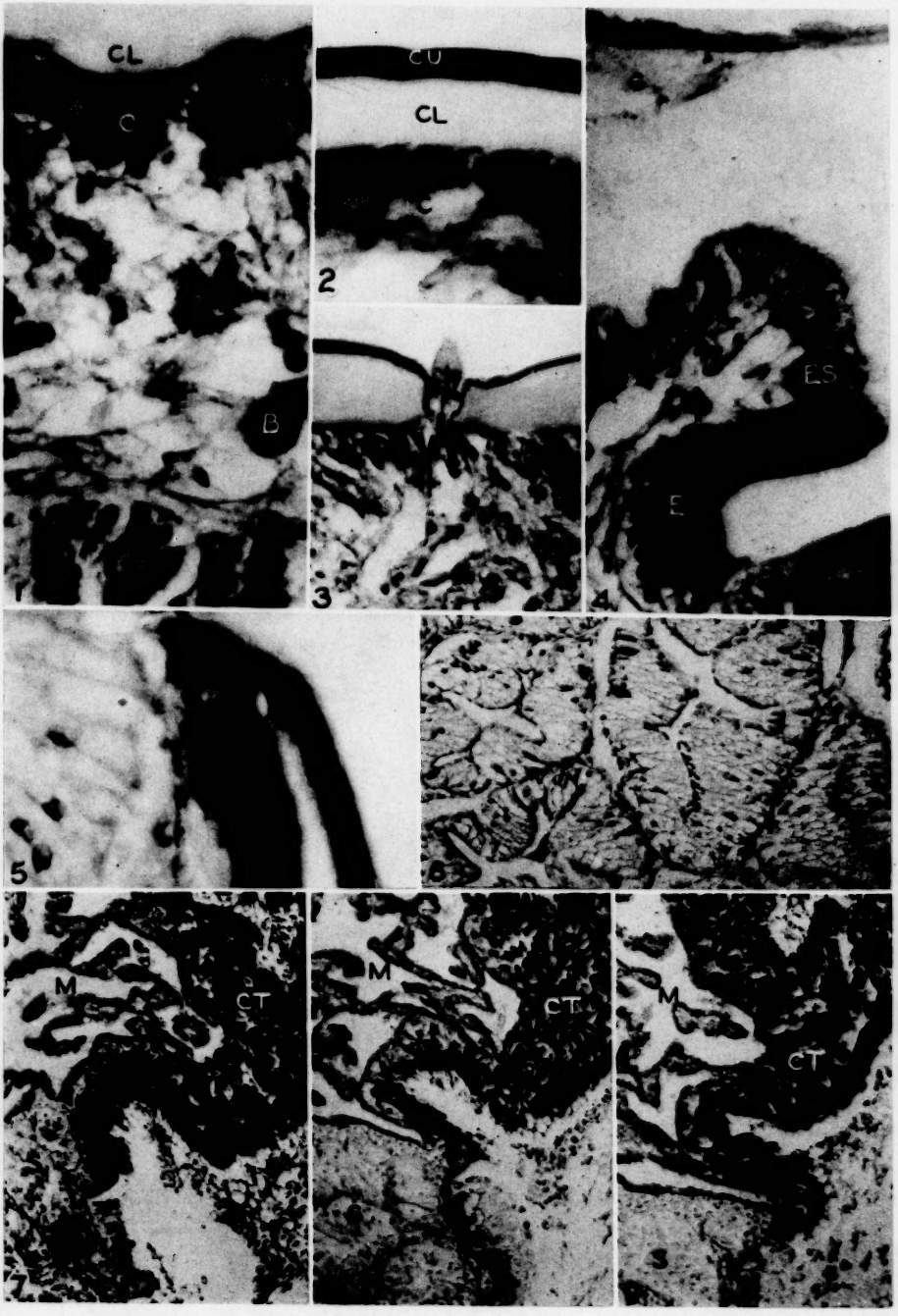
FIG. 6.—Digestive gland of molting crayfish. Cross-section. Incubated 72 hours with fructose diphosphate. ×75. At molting, the gland is weakly positive for alkaline phosphatase.

FIG. 7.—Nephridium of premolt crayfish. Incubated 72 hours with fructose diphosphate. ×75.

FIG. 8.—Nephridium of premolt crayfish. Incubated 5 hours with adenylic acid. ×75. The cuticuloid borders and basement membranes of the cortical epithelium display strong alkaline phosphatase reaction.

FIG. 9.—Nephridium of premolt crayfish. Incubated 24 hours with adenylic acid. ×75. Compared with Fig. 8, the longer incubation period has resulted in a pronounced phosphatase reaction throughout the cytoplasm of the cortical epithelium.

PLATE I



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THE LIPID COMPOSITION OF TENEBRIO MOLITOR LARVAE[1]

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PATTERNS of morphological change during development have long claimed the attention of biologists. In recent years there has been an added interest in patterns of changing chemical composition during embryonic and larval growth. Various schemata have been designed to establish order in and meaningfulness to the observed data. Apart from simple graphs relating chemical changes to morphological events during development, the most inclusive general system of curves has probably been that which describes heterogonic growth. This concept was first applied on a comprehensive scale by Huxley (1924, 1932) to relative morphological growth and soon thereafter by Teissier (1931) and Needham (1934) to the changing chemical constituents in the growing organism.

Briefly, the family of curves illustrating heterogonic growth, later termed "allometric" by Huxley and Teissier (1936) and "heterauxetic" by Needham and Lerner (1940), is based on the parabola $y = bx^k$, in which y represents the magnitude of the component part, x represents the total organism, and b and k are constants. In logarithmic form this becomes $\log y = \log b + k \log x$. If, when the data are plotted on a double-log grid, the points reasonably fall along a straight line, growth is said to be "heterogonic" or "heterauxetic." The value of k gives the slope of this line and, as such, becomes indicative of whether the ratio of the rate of growth of the component part is greater than ($k > 1.00$), equal to ($k = 1.00$), or less than ($k < 1.00$) that of the whole. The constant b is less significant, since it merely gives the elevation of the line, or the value of y when x is unity. Needham (1942) recently summarized a large volume of information on the changing chemical patterns during the development of various organisms by expressing the data in terms of k, the constant of relative growth, thereby affording a comparison which widely crosses species lines.

This "differential growth ratio," as Huxley termed it, simply means that empirically an increase of 1 per cent in x is associated with an increase of k per cent in y. Does such a constant change when the organism is subjected to varying external conditions during growth? Is it an expression of a relatively immutable chemical pattern, or does it require a further formulation in order to describe its behavior when systematically varying stresses are imposed on the organism? The present study, which deals with the pattern of lipid composition of the meal worm larva, *Tenebrio molitor* L., raised under different temperatures, was undertaken in an attempt to obtain an answer to these questions.

Chemical heterauxesis was studied extensively in *Tenebrio* by Teissier (1931) but not under differing conditions. He found, for example, that the amount of total fat could be related to the total fresh weight by the expression $y = 0.115\, x^{1.13}$. The value of k, 1.13, indicates

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that the rate of accumulation of fat occurs more rapidly than that of the total body weight. Similar tachyauxetic values ($k > 1.00$) have also been found for the total fat composition of the waxmoth, rat, and chick (Needham, 1942).

Romanoff and Faber (1933) investigated the growth and fat content of White Leghorn chick embryos raised at five different temperatures from 32° to 40° C. from the 16th day to hatching. Using these data, Needham (1942) calculated the k-values for fat plotted against wet weight and found that, for the four lower temperatures, the values were essentially uniform (1.77–1.84). This study was necessarily limited by the short time interval and hence the small amount of growth, as well as by the narrowness of the range of temperatures tolerated by that organism. A poikilothermic animal, such as the meal worm, offers a wider range of permissible temperatures and, in addition, allows observations over a longer period of time (over 200 days) and over a many-fold increase in weight. Moreover, it is an animal which is very easily reared.

The lipid composition of *Tenebrio* larvae was studied at approximately 20°, 25°, and 30° C. at 25-day intervals. Total lipid, neutral fat, total fatty acids, phospholipid, and cholesterol analyses were made in the course of larval development. The data obtained from these determinations were then analyzed for heterauxetic growth.

MATERIAL AND METHODS

The meal worm larvae were raised in glass dishes measuring 20–24 cm. in diameter and 6 cm. in height, with the culture medium 3–4 cm. deep. This arrangement provided adequate surface for aeration and rapid equilibration of temperature. The food consisted exclusively of bran, which proved to be nutritionally adequate to carry the insects to the adult stage. Waste products, such as excreta, exuviae, and the finely ground "conditioned" bran, were removed, and fresh bran was added at frequent intervals.

Cultures were started by placing an identical number of adults in each of three culture dishes, one for each temperature. The adults were removed after 1 week, and the middle of the week was taken as the starting day for the culture. This procedure involved a small but probably unimportant error, inasmuch as no correction was made for the length of time from egg-laying to hatching. Eggs were laid at the temperatures at which the larvae were maintained.

Culture dishes were placed, uncovered, in incubators, which were kept at roughly 20°, 25°, and 30° C. Table 1 gives the values for the temperature readings in the bran and in the air alongside the dishes. Readings were made almost daily during the period from July 19, 1945, to June 17, 1946, during which period the cultures reported here were raised. The coefficients of variation for these readings ranged from 2.2 to 3.5 per cent and averaged 2.9 per cent. No attempt was made to control the relative humidity, except to insure the absence of very dry conditions by placing a dish of water in each incubator. Approximate relative-humidity readings were made periodically in each of the incubators by means of a dial-type hygrometer. Data derived from these readings are also summarized in Table 1. It is evident that the variations were rather large but that a middle range of humidity was maintained.

At 25-day intervals groups of larvae were removed from each culture, counted, brushed free of adherent particles of medium, and weighed. A sufficient number of larvae were taken to give a fresh

(=wet) weight of at least 0.3 gm. and in most cases of over 1.0 gm. Of the 49 samples, 3 were less than 0.5 gm., and 38 were between 1.0 and 3.0 gm. The number of larvae comprising each sample varied inversely with their size: from 6 to 30 larvae were used when they weighed between 75 and 127 mg., while 100–300 were used when the individual weights ranged from 1.8 to 5.0 mg.

The larvae were placed, after being weighed, in small flasks and covered with 10 ml. of 95 per cent alcohol. Within several days this alcohol was decanted and filtered through fat-free paper (Whatman No. 43) into a 100-ml. volumetric flask. The larvae were then ground with a small amount of sea-sand in a mortar, and the sand-tissue mixture was quantitatively transferred to a thimble of the fat-free filter paper, which was placed in a 200-ml. Underwriter extractor. The material was extracted twice over a hot plate, each time for 15 minutes with fresh 30-ml. portions of a 3:1 alcohol-ether mixture, which was then filtered into the volumetric flask and made up to volume with 95 per cent alcohol. From this extract duplicate determinations were made of total fatty acids, phospholipid, and total and free cholesterol.

Separation of these component lipids was accomplished largely by the procedures outlined by Boyd (1938) and Bloor (1943). Determinations were done by the dichromate–sulphuric acid oxidation method.

Total fatty acids and total cholesterol were first separated by using a 10–25-ml. aliquot, which was pipetted into a 125-ml. Erlenmeyer flask and to which 5 drops of 40 per cent NaOH were added. This was slowly evaporated on a steam bath so that approximately 1 ml. was left after 1 hour. Two to three drops of 0.04 per cent phenol red, followed by 0.2 ml. of 25 per cent sulphuric acid, were added. The mixture was then extracted five or six times with small portions of petroleum ether, which were decanted carefully from the red aqueous portion into a 50-ml. flask and made up

TABLE 1

SUMMARY OF TEMPERATURE AND RELATIVE-HUMIDITY DATA

	20° C.		25° C.		30° C.	
	Flour	Air	Flour	Air	Flour	Air
	Temperature					
Mean	19.69	19.98	24.48	25.05	30.12	30.16
Standard error	0.044	0.038	0.039	0.036	0.060	0.038
Coeff. of var. (%)	3.50	3.16	2.74	2.38	3.39	2.18
	Relative Humidity (Per Cent)					
Mean	63.6		45.9		46.7	
Standard error	0.56		0.64		0.67	
Coeff. of var. (%)	8.0		11.4		13.6	
Vapor tension (mm.)	11.0		10.8		14.7	
Saturation deficit (mm.)	6.3		12.7		16.3	

to volume. An aliquot of 2–5 ml. of this was pipetted into a 125-ml. Erlenmeyer flask, evaporated to dryness over steam, gently ventilated with an air stream, and used immediately for oxidation to determine total fatty acids plus total cholesterol. The latter was subtracted, after independent determination, to give a value for total fatty acids alone.

Cholesterol was separated by a combination of the methods of Kelsey (1939) and Boyd (1938). For total cholesterol, aliquots, usually of 20 ml., of the petroleum ether extract were evaporated over steam in flasks down to a few milliliters and were transferred to 15-ml. centrifuge tubes. The flasks were rinsed twice with petroleum ether, and these washings were added to the tubes. The contents of the latter were then gently evaporated, with the aid of boiling-rods, down to ½ ml. in a hot-water bath. Two ml. of acetone were added according to the suggestion of Hunter *et al.* (1945), and the contents were again evaporated to ½ ml. Digitonin, 4 ml. of a 0.2 per cent solution in 95 per cent alcohol, was added and was followed by three drops of 10 per cent hydrochloric acid (Popjak, 1943), and the tubes were stored overnight in the icebox. They were then centrifuged, and the supernatant solvent was discarded. Remaining lipids other than the cholesterol-digitonide precipitate were removed by rinsing twice with petroleum ether, centrifuging, and decanting. From this point on, a modification of Boyd's method was used. The precipitate was dissolved in 5 ml. of absolute methyl alcohol, which was brought to a boil and decanted into the oxidation flasks. This procedure was then repeated with a second 5-ml. portion of methyl alcohol. The combined methanol washings were evaporated over steam, dried with an air stream, and immediately oxidized.

For the separation of the phospholipid and free-cholesterol fractions, 25-ml. aliquots of the alcohol-ether extracts were placed in 125-ml. Erlenmeyer flasks and evaporated to a few milliliters on a hot plate. The boiling-rod was rinsed with a few drops of petroleum ether and was removed, and the evaporation was completed over steam. A very brief, gentle stream of air was used to blow out any remaining vapors. The residue was immediately extracted three times with small amounts of petroleum ether, which were decanted into a 15-ml. centrifuge tube. In several instances particles of the residue were carried into the centrifuge tubes by the petroleum ether. Such tubes were centrifuged, decanted to fresh tubes, and rinsed with fresh petroleum ether, which was likewise added to the clean tube. After these ether washings were concentrated to ½ ml. by gently boiling the tubes in a hot-water bath, 7 ml. of acetone and 0.1 ml. of 30 per cent magnesium chloride in 95 per cent alcohol were added. The tubes were allowed to stand overnight to obtain a maximum precipitation of the phospholipids. The acetone, containing the free cholesterol, was decanted to fresh tubes, and the residue was rinsed with a small amount of fresh acetone, which, after being centrifuged, was added to the first portion.

The phospholipid was further treated by dissolving the acetone precipitate in 5 ml. of chloroform, which was heated to boiling and poured into an oxidation flask. The tube was rinsed with 2–3 ml. of chloroform, which was brought to boiling and was added to the contents of the oxidation flask. The flasks were evaporated on a steam bath and blown gently with a brief stream of air; the oxidizing agents were added directly.

Free cholesterol was separated by evaporating the phospholipid-free acetone to ½ ml. From this point on, the

samples were treated exactly as were the total cholesterol fractions, i.e., by precipitation with digitonin and three drops of 10 per cent hydrochloric acid.

Oxidation was accomplished by adding 5.0 ml. of Nicloux reagent and 3.0 ml. of approximately N potassium dichromate (Boyd, 1938). The flasks were then placed in an oven at 124° C. for 20 minutes, after which 25 ml. of cold distilled water were added. When the flasks had cooled to room temperature, 10 ml. of freshly prepared 10 per cent potassium iodide were added, and the solutions were titrated with standardized 0.02 N sodium thiosulphate, with 1 per cent starch indicator. Blanks consisting of Nicloux reagent and potassium dichromate were also prepared, heated in the oven with the other flasks, and titrated at this point. Blanks prepared by evaporating comparable portions of the solvents gave results identical with those made up of oxidizing agents alone.

Each item of glassware employed in the extraction, separation, and titration procedures was carefully cleaned before use by means of sulphuric acid–dichromate solution, was thoroughly rinsed in distilled water, and allowed to drain dry. All chemicals used were of reagent grade. Ether was rendered peroxide-free just before use in extraction by shaking it with freshly prepared 10 per cent potassium iodide and rinsing it several times with distilled water.

Results were calculated according to the outline given by Boyd (1938). From the determinations of total fatty acid, phospholipid, and total and free cholesterol, the following were estimated: cholesterol ester fatty acids, phospholipid fatty acids, triglyceride fatty acids, and neutral fat. The total lipid consists of the sum of neutral fat, phospholipid, total cholesterol, and cholesterol fatty acids. The various values for each extract were then converted to grams per 1,000 larvae (mg/larva).

Duplicate determinations were made, and the median percentage agreement between these ranged from 2.4 per cent for total fatty acids to 11.2 per cent for total cholesterol; the values for free cholesterol and phospholipid were 5.3 and 5.5 per cent, respectively. Statistical evaluation of the results was done according to standard methods, referred to in detail later. *P*-values were derived from tables of "Student's" distribution (1925), and 0.05 was taken as the upper (5 per cent) level of significance.

EXPERIMENTAL RESULTS

GROWTH OF LARVAE

The growth data for the three cultures which were followed most completely are given in Table 2. It will be seen that there is considerable variation among cultures at any one temperature. In order to have some measure of this variability, triplicate samples of equal numbers were weighed for Series III. Lipid analysis was done on one of these samples, and the others were returned to the culture. Since the standard error of a mean is the standard deviation of a sampling distribution of means, the latter statistic was calculated from the means of the three samples. The standard deviation of a single sample of larvae was then estimated by a reversal of the usual calculation, namely, by multiplying the standard error of the mean (calculated as the standard deviation of the three sample means) by $\sqrt{n}$ of the sample. This value and the coefficient of variation obtained by dividing it by the mean are given for each of the several age and temperature levels in the second part of the table. That there is a marked variation among cultures is not surprising, in view of the variation from sample to sample within a single culture.

TABLE 2

GROWTH OF LARVAE AT DIFFERENT TEMPERATURES

DAYS	WEIGHT (MG.) PER LARVA							
	Culture I (Mean)	Culture II (Mean)	Culture III (Mean)	Total (Mean)	Variation in Culture III			
					No.	Standard Error	Standard Deviation	Coeff. of Variation (%)
	20° C.							
50	2.04	.....	1.86	1.95	100	0.0596	0.596	32.04
75	4.61	6.47	5.19	5.42	50	0.1556	1.100	21.20
100	15.53	22.82	.....	19.18	.....	.....	.....	.....
125	48.34	40.70	.....	44.52	.....	.....	.....	.....
150	94.74	82.95	92.21	89.97	7	6.5079	17.218	18.67
175	.....	108.72	.....	108.72	.....	.....	.....	.....
200	.....	.....	127.03	127.03	.....	.....	.....	.....
Mean	.....	.....	.....	.....	.....	.....	.....	23.97
	25° C.							
50	3.04	3.46	2.78	3.09	100	0.1046	1.046	37.67
75	9.76	12.42	8.40	10.19	50	1.2720	8.994	107.10
100	30.73	36.20	21.52	29.48	20	1.3497	6.036	28.05
125	57.15	49.61	.....	53.38	.....	.....	.....	.....
150	91.57	81.20	49.74	74.17	20	0.4781	2.138	4.30
175	95.71	92.61	.....	94.16	.....	.....	.....	.....
200	100.63	.....	89.08	94.86	12	2.4414	8.457	9.66
220	.....	.....	110.81	97.69	20	6.3640	28.461	24.66
225	.....	84 56	.....		.....	.....	.....	.....
250	.....	93.52	.....	93.52	.....	.....	.....	.....
Mean	.....	.....	.....	.....	.....	.....	.....	35.24
	30° C.							
50	7.21	8.22	7.54	7.66	75	0.3022	2.617	34.68
75	25.51	24.43	16.16	22.03	40	1.2076	7.638	47.28
100	50.92	34.99	16.76	34.22	20	1.9277	8.621	51.45
125	51.84	39.12	.....	45.48	.....	.....	.....	.....
150	59.64	58.29	49.29	55.74	20	2.9167	13.044	26.46
175	64.84	53.11	.....	58.98	.....	.....	.....	.....
200	63.73	.....	.....	63.73	.....	.....	.....	.....
225	.....	44.17	.....	44.17	.....	.....	.....	.....
250	49.12	40.91	.....	45.02	.....	.....	.....	.....
275	44.52	53.94	.....	49.23	.....	.....	.....	.....
300	48.01	.....	.....	48.01	.....	.....	.....	.....
Mean	.....	.....	.....	.....	.....	.....	.....	39.97

The mean growth curves of the larvae at each of the three temperatures are plotted in Figure 1. It is apparent that the larvae at the lower temperatures grew faster, after a slow start, and reached pupation earlier than did those at the higher temperatures. These curious results are undoubtedly referable to the differences in relative humidity, for

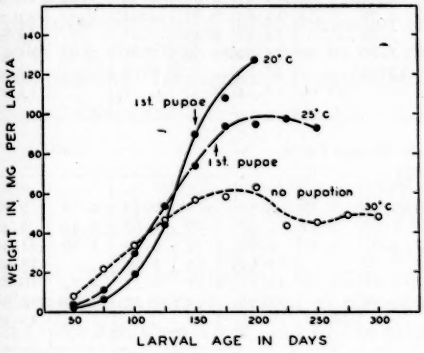

FIG. 1.—Growth of meal-worm larvae at different temperatures.

interpolation of these curves with the data given by Menusan (1936) gives a rather consistent picture (Fig. 2). It is quite evident that humidity is at least as important as is temperature in influencing the larval growth of this organism.

LIPID COMPOSITION OF LARVAE

Data on lipid composition are presented in Table 3 for each of the three series which were analyzed in detail. These constitute the basic information of this paper. Data have been restricted to 200 days, since either pupation was well under way at this time (20°, 25° C.) or a maximum in the growth curve had been reached (25°, 30° C.). For each value in the table the percentage of the wet weight was calculated, and the means of these have been included in the table.

Apart from occasional variations, there is a general consistency in the trends shown by the results. As larval growth progresses, the total lipid becomes an increasing proportion of the body weight (Table 3, A). Neutral fat, as the major component of the total lipid, similarly increases with respect to total weight, as do the total fatty acids (Table 3, B, C). Total cholesterol, on the other hand, while it is increased in amount with larval growth, maintains a nearly constant proportion to the total weight. The mean per cent wet-weight values for this component vary only be-

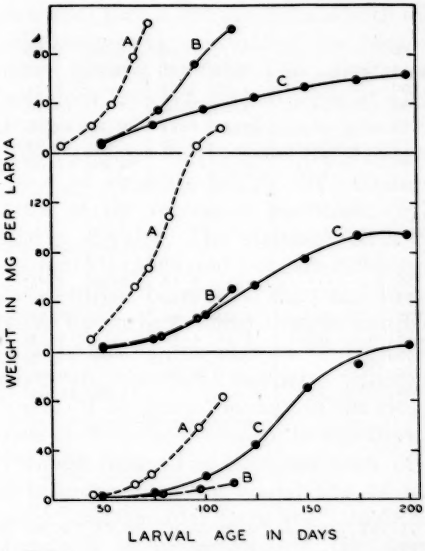

FIG. 2.—Growth of meal-worm larvae at different temperatures and humidities.

tween 0.11 and 0.32 per cent, except at 175 days (Table 3, D). The picture with this component is complicated by the unusually large values at all temperatures in Series III at 150 days and in Series II at 175 days. These discrepancies are traceable to departures from the procedures for separation of total cholesterol which occurred only in these in-

TABLE 3

LIPID COMPOSITION OF MEAL WORMS IN GRAMS PER 1,000 LARVAE

AGE IN DAYS	20° C.				25° C.				30° C.			
	Culture			Mean Per Cent of Wet Weight	Culture			Mean Per Cent of Wet Weight	Culture			Mean Per Cent of Wet Weight
	I	II	III		I	II	III		I	II	III	
	A. Total Lipid											
50	.....	0.14	0.18	8.36	.....	0.24	0.24	7.79	.....	0.68	0.74	9.05
75	0.49	0.47	0.49	9.10	1.04	1.14	0.84	10.01	2.96	2.54	1.62	10.69
100	1.44	2.30	.....	9.65	3.39	4.38	2.54	11.65	6.87	4.92	2.12	13.41
125	5.83	4.82	.....	11.95	8.01	7.22	.....	14.28	8.42	5.91	.....	15.67
150	11.73	9.66	11.84	12.29	14.43	12.10	6.07	14.29	10.59	7.01	3.03	11.97
175	.....	11.22	.....	10.32	.....	13.64	.....	14.73	.....	6.11	.....	11.49
200	.....	.....	16.38	12.90	15.02	.....	16.69	17.24	8.37	.....	.....	13.13
	B. Neutral Fat											
50	.....	0.09	0.14	5.86	.....	0.16	0.18	5.51	.....	0.53	0.62	7.79
75	0.35	0.30	0.39	6.57	0.73	0.80	0.72	7.48	2.26	1.87	1.42	8.44
100	1.07	1.77	.....	7.32	2.67	3.63	2.21	9.67	5.77	4.12	1.86	11.39
125	5.23	3.86	.....	10.16	7.34	6.20	.....	12.67	7.83	5.00	.....	13.94
150	10.47	8.52	10.43	10.88	13.37	11.31	5.46	13.17	9.71	6.32	2.33	10.62
175	.....	9.38	.....	8.63	.....	11.99	.....	12.95	.....	5.34	.....	10.06
200	.....	.....	14.82	11.66	13.83	.....	16.01	16.25	7.88	.....	.....	12.36
	C. Total Fatty Acids											
50	.....	0.12	0.16	7.11	.....	0.20	0.21	6.63	.....	0.59	0.66	7.98
75	0.41	0.39	0.43	7.74	0.88	0.98	0.76	8.62	2.54	2.20	1.47	9.35
100	1.24	2.00	.....	8.38	2.98	3.90	2.30	10.38	6.16	4.41	1.92	12.04
125	5.31	4.27	.....	10.74	7.36	6.53	.....	13.02	7.78	5.32	.....	14.31
150	10.68	8.77	10.64	11.13	13.33	11.19	5.44	13.09	9.75	6.40	2.54	10.83
175	.....	9.89	.....	9.09	.....	12.21	.....	13.19	.....	5.44	.....	10.24
200	.....	.....	15.01	11.82	13.86	.....	15.58	16.02	7.77	.....	.....	12.18
	D. Total Cholesterol											
50	.....	0.01	0.00	0.23	.....	0.01	0.00	0.22	.....	0.02	0.01	0.19
75	0.02	0.01	0.01	0.30	0.05	0.02	0.02	0.31	0.14	0.04	0.03	0.30
100	0.04	0.05	.....	0.24	0.07	0.09	0.05	0.24	0.11	0.07	0.04	0.22
125	0.11	0.06	.....	0.18	0.10	0.07	.....	0.16	0.10	0.05	.....	0.17
150	0.20	0.16	0.43	0.29	0.13	0.15	0.32	0.32	0.09	0.12	0.28	0.31
175	.....	0.51	.....	0.47	.....	0.58	.....	0.63	.....	0.30	.....	0.57
200	.....	.....	0.21	0.17	0.11	.....	0.15	0.14	0.07	.....	.....	0.11
	E. Phospholipid											
50	.....	0.04	0.04	2.23	.....	0.07	0.05	2.06	.....	0.14	0.11	1.54
75	0.11	0.16	0.09	2.16	0.24	0.32	0.11	2.11	0.49	0.62	0.17	1.84
100	0.32	0.47	.....	2.05	0.62	0.64	0.27	1.69	0.96	0.73	0.22	1.75
125	0.46	0.90	.....	1.58	0.54	0.94	.....	1.73	0.45	0.86	.....	1.53
150	0.99	0.94	0.76	1.00	0.89	0.59	0.13	0.65	0.77	0.74	0.26	0.91
175	.....	1.08	.....	0.99	.....	0.74	.....	0.80	.....	0.30	.....	0.56
200	.....	.....	1.26	0.99	1.06	.....	0.48	0.81	0.42	.....	.....	0.66

stances. Probable values can be estimated from the free-cholesterol determinations, which were not affected. For each temperature the mean per cent that free cholesterol constituted of the total cholesterol, with its standard deviation, follows: for 20°, 56.4 ± 8.9 per cent; for 25°, 64.7 ± 5.0 per cent; for 30°, 66.4 ± 6.2 per cent. When these percentages are used to estimate values of the total cholesterol, more consistent results are obtained, as shown in the accompanying tabulation. Phospholipid,

Days	20°	25°	30°
150	0.20%	0.17%	0.17%
175	0.22%	0.17%	0.15%

in contrast to neutral fat, declines very slowly in percentage, so that at 200 days it forms less than 1 per cent of the total weight, while at 50 days it constitutes from 1.5 to 2.2 per cent (Table 3, E).

PERCENTAGE COMPOSITION OF TOTAL LIPIDS

These relations are made more striking by a consideration of the changing composition of the lipids as such. When the values are calculated in terms of percentage of total lipid, the trends shown in Table 4 are seen. Neutral fat becomes an increasingly greater component of the total lipid as larval development progresses. Phospholipids, contrariwise, show a fairly steady decline in this regard, although, as has been seen, they maintain a small but almost constant percentage of the wet weight of the organism. The total fatty acids combine both these trends by a slowly but consistently increasing share of the total lipid composition. Total cholesterol shows practically no change in its proportion to total fat; the values at 150 and 175 days, as has been explained, are probably incorrectly large. In Figure 3, in which the various components are plotted, the estimated values based on the free-cholesterol determinations have been used.

HETERAUXESIS

The data were next analyzed for allometry. Figure 4 shows the double-log plot of total lipids against wet weight for each of the temperatures. Since the points show a fairly good distribution along a straight line, they may be said to behave heterauxetically. Values of k were calculated by fitting a least-squares straight line to the logarithms of the weights. The regression coefficient of such a line is identical with the relative growth constant of the heterogonic growth formula. This calculation was done for each culture series at each temperature. The results are given in Table 5, which also includes the standard error of estimate ($\overline{S}_{y \cdot x}$), the standard error of the regression coefficient (σ_k), and a P-value. The standard error of estimate is calculated from the difference between the observed values and those given by the best-fitting straight line and is adjusted for the number of points to give an unbiased estimate (Ezekiel, 1941). It is thus a measure of the closeness of fit of the points to the line drawn through them. The standard error of k is a measure of the variability of the slope as estimated from the sample at hand. It is interpreted in the same manner as is the standard error of a mean. The P-value given in Table 5 is derived from the t-test of significance, which evaluates the difference between the observed k-value and a theoretical value (Smith, 1939). In this instance a theoretical value of 1.00 was selected because it represents a rate of growth identical with that of the total. The P-values, obtained from "Student"'s distribution for small numbers, give a measure of the significance of the departures

of the observed values of k from the isauxetic value of 1.00. These statistical indices were then calculated for the combination of the three series for each temperature to give over-all values at each of the temperatures and again for all the data combined.

Of the nine elements in Table 5, where the series and temperatures are considered individually, five show a statistically significant increase in the k-values over 1.00, and these instances are all at 20° and 25° C. When the three series are combined at each temperature, the values of k range from 1.098 at 20° to 1.202 at 25°, each of which departs significantly from 1.00. However, there is an intermediate value of 1.165 at 30°, which is not significant because of the greater scatter of the points about this line. The greater variation at 30° is indicated by the higher value of $\overline{S}_{y \cdot x}$ at this temperature. The results of the t-tests for significance of the differences between the k-values of the several temperatures are given in Table 6, A, where it can be seen that only the 20° versus 25° comparison yields a significant difference.

The values of k have been subjected to a further evaluation by an analysis

TABLE 4

LIPID DISTRIBUTION IN MEAN PERCENTAGE OF TOTAL LIPID

Days	Neutral Fat	Phospho-lipid	Total Cholesterol	Cholesterol Ester Fatty Acid	Total Lipid	Total Fatty Acids
	20° C.					
50	68.89	27.61	2.85	0.65	100.00	84.90
75	71.50	24.49	3.31	0.69	99.99	84.97
100	75.78	21.23	2.46	0.54	100.01	86.76
125	84.92	13.29	1.52	0.28	100.01	89.86
150	88.51	8.22	2.31	0.95	99.99	90.55
175	83.61	9.62	4.54	2.24	100.01	88.10
200	90.44	7.71	1.31	0.54	100.00	91.63
	25° C.					
50	70.14	26.82	2.77	0.27	100.00	84.82
75	74.75	21.27	3.05	0.94	100.01	86.14
100	82.82	14.60	2.05	0.53	100.00	88.99
125	88.78	9.94	1.12	0.17	100.01	90.87
150	92.02	4.38	2.47	1.12	99.99	91.48
175	87.95	5.42	4.26	2.36	99.99	89.54
200	94.04	4.97	0.82	0.18	100.01	92.85
	30° C.					
50	80.28	17.20	2.15	0.38	100.01	88.14
75	79.19	17.15	2.70	0.95	99.99	87.63
100	85.01	13.00	1.64	0.35	100.00	89.82
125	88.81	9.95	1.05	0.19	100.00	91.23
150	86.27	7.84	3.94	1.93	99.98	89.15
175	87.46	4.83	4.95	2.74	99.98	89.08
200	94.12	5.00	0.84	0.02	99.98	92.79

of variance by which the variation among cultures and among temperatures are tested together. This can be done, since the numbers of observations in each cell are approximately equal. The formulae given by Goulden (1939) were used for this purpose, and the results are given in Table 7, A. The *F*-values so obtained are far below that for the 5 per cent level

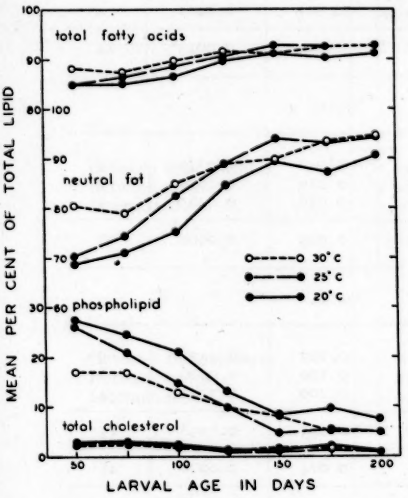

FIG. 3.—Changes with age of mean percentage of total lipid of various components.

of significance (6.94) and hence may be taken to show no significant variation among temperatures or among experiments.

Teissier reported a k of 1.13 for his data of total fat against total wet weight. Recalculation from his published data by the least-squares method used here gives a value of 1.117 with a standard error (σ_k) of 0.024. This standard error is the same as that obtained by grouping all the data in the present paper, for which k is 1.149. The good agreement between these two k-values is shown by the lack of a statistically significant difference, since the t-test gives a ratio of 0.93.

Similar analyses can be made for the several lipid components in relation to the total lipid. Its major constituent, neutral fat, shows good heterauxesis with respect to the total (Fig. 5). The data are summarized in Table 8, A. That these values fit a straight line even more closely than do those of the total lipid against wet weight is indicated by their smaller standard error of estimate. The k-values for each of the temperatures, although very close to 1.00, also depart signifi-

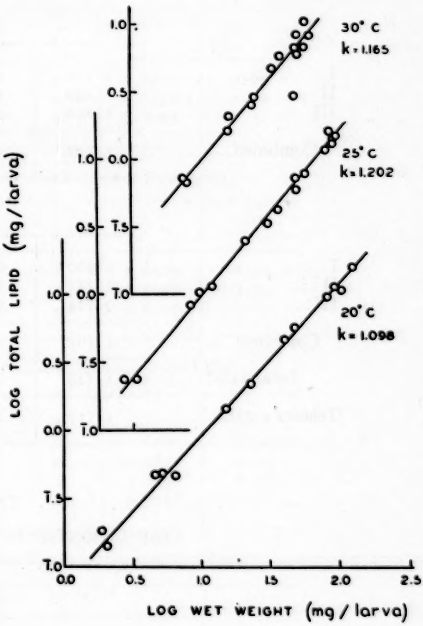

FIG. 4.—Double-log plot of total lipid against wet weight at various temperatures.

cantly from isauxesis, and this situation is especially true for the two lower temperatures. None of the comparisons between temperatures shows a significant difference by the t-test (Table 6, B). Analysis of variance (Table 7, B) reveals practically no differences among temperatures and quite unimportant differences among experiments.

TABLE 5

HETERAUXETIC CONSTANTS FOR TOTAL LIPID IN RELATION TO WET WEIGHT

SERIES	HETERAUX. CONSTANT k	S.E. OF ESTIMATE $S_{y \cdot x}$	S.E. OF REGRESS. COEFF. σ_k	P	n
	20° C.				
I	1.070	0.052	0.052	0.3152	4
II	1.136	0.049	0.036	0.0197	6
III	1.078	0.026	0.017	0.0421	4
Combined	1.098	0.057	0.024	0.0015	14
	25° C.				
I	1.170	0.035	0.042	0.0273	5
II	1.240	0.023	0.019	0.0000	6
III	1.202	0.067	0.056	0.0365	5
Combined	1.202	0.045	0.023	0.0002	16
	30° C.				
I	1.300	0.066	0.207	0.2458	5
II	1.221	0.070	0.100	0.0926	6
III	0.724	0.116	0.200	0.3044	4
Combined	1.165	0.110	0.095	0.1058	15
Total data	1.149	0.080	0.024	0.0000	45
Teissier's data	1.117	0.033	0.024	0.0006	12

TABLE 6

COMPARISONS OF HETERAUXETIC CONSTANTS

	20° vs. 25°		20° vs. 30°		25° vs. 30°	
	t	P	t	P	t	P
A. $\frac{\text{Total lipid}}{\text{Wet weight}}$	3.15	0.0040	0.69	Not signif.	0.38	Not signif.
B. $\frac{\text{Neutral fat}}{\text{Total lipid}}$	0.60	Not signif.	0.50	Not signif.	0.92	Not signif.
C. $\frac{\text{Phospholipid}}{\text{Total lipid}}$	1.39	Not signif.	0.43	Not signif.	0.54	Not signif.
D. $\frac{\text{Total cholesterol}}{\text{Total lipid}}$	1.23	Not signif.	0.74	Not signif.	0.20	Not signif.
E. $\frac{\text{Total fatty acid}}{\text{Total lipid}}$	0.41	Not signif.	0.11	Not signif.	0.36	Not signif.

The decline in percentage composition of phospholipid with respect to the total lipid as development proceeds is reflected in the mean k-values, which range from 0.559 to 0.704 for the several temperatures (Table 8, B). That there is considerable scatter of points about the straight lines can be seen in Figure 6 and from the $\overline{S}_{y \cdot x}$ values, which are larger than those in the two categories already

TABLE 7

ANALYSIS OF VARIANCE

	Sum of Squares	Degrees of Freedom	Variance σ^2	F*
A. Total Lipid versus Wet Weight				
Total	0.2273	8	.....	.....
Between temperatures	0.0390	2	0.0195	0.67
Between series	0.0712	2	0.0356	1.22
Interaction	0.1171	4	0.0293	.....
B. Neutral Fat versus Total Lipid				
Total	0.0241	8	.....	.....
Between temperatures	0.0006	2	0.0003	0.14
Between series	0.0154	2	0.0077	3.79
Interaction	0.0081	4	0.0020	.....
C. Phospholipid versus Total Lipid				
Total	0.2511	8	.....	.....
Between temperatures	0.0948	2	0.0474	2.24
Between series	0.0718	2	0.0359	1.70
Interaction	0.0845	4	0.0211	.....
D. Total Cholesterol versus Total Lipid				
Total	1.5842	8	.....	.....
Between temperatures	0.1494	2	0.0747	0.54
Between series	0.8907	2	0.4454	3.27
Interaction	0.5441	4	0.1360	.....
E. Total Fatty Acid versus Total Lipid				
Total	0.0046	8	.....	.....
Between temperatures	0.0000	2	0.0000	0.00
Between series	0.0027	2	0.0014	2.78
Interaction	0.0019	4	0.0005	.....

* For comparisons of variance of temperature and of series with interaction, the F-values for the 5 and 1 per cent levels of significance, respectively, are 6.94 and 18.00.

discussed. In spite of this variation the *P*-values indicate that the heterauxetic constants again depart significantly from 1.00, especially at the lower temperatures. Here, too, the differences between temperatures are not significant by direct comparison (Table 6, C) or by the analysis of variance (Table 7, C).

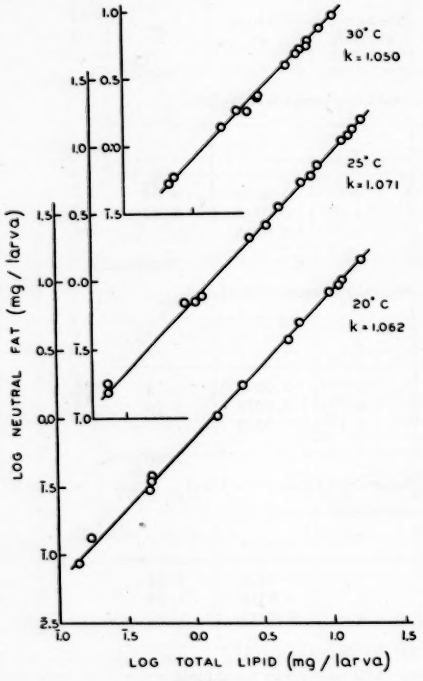

FIG. 5.—Double-log plot of neutral fat against total lipid at various temperatures.

Teissier (1931) reported a k of 0.83 for lipid phosphorus against wet weight. Calculation of k by least squares from his data gives a value of 0.844, with a standard error of estimate of 0.029 and a σ_k of 0.024. Since lipid phosphorus constitutes an almost constant fraction (approximately 1/26) of phospholipid, the relative rates of increase of these two substances should be identical when measured simultaneously. In the present investigation the over-all value of k, for all temperatures combined, for phospholipid against total lipid is 0.628. This can be converted to the value for phospholipid versus wet weight by multiplying by the value for total lipid versus wet weight, viz., $0.628 \times 1.149 = 0.722$. The latter value is somewhat lower than that found by Teissier but is roughly similar to it.

The data for total cholesterol show a fit to rectilinearity comparable to that for phospholipid, since the standard errors of estimate for these two components are of the same magnitude. In calculating the regression coefficients for total cholesterol, the values of Series III at 150 days and of Series II at 175 days have been omitted. Their wide divergence from the bulk of the data can be appreciated from Figure 7, where they are indicated by closed circles; and the inclusion of these data in the calculations produces standard errors of estimate which are quite large. The k-values given in Table 8, C, are less than 1.00, and they depart from it significantly at the lower temperatures and almost so at 30°. They do not differ from one another significantly (Table 6, D). Analysis of variance reveals no significant differences among temperatures or among series.

The final component analyzed by these methods was that of total fatty acids, which comprise 95 per cent of the neutral fat and 67 per cent of the phospholipid, plus the small amount of cholesterol-ester fatty acids. When the total fatty acids are plotted against the total lipid, the points show an exceedingly good fit to a straight line (Fig. 8), and calculations demonstrate a consistent and unusually small standard error of estimate at each of the temperatures (Table 8, D). Although the slope of the line at each temperature is quite close to 1.00, it departs from it very significantly at the

TABLE 8

HETERAUXETIC CONSTANTS FOR COMPONENTS OF TOTAL LIPID

	Temperature						Grand Total	
	20° C.		25° C.		30° C.			
	n	*k*	*n*	*k*	*n*	*k*	*n*	*k*
A. Neutral Fat versus Total Lipid								
I	4	1.079	5	1.109	5	1.168		
II	6	1.083	6	1.089	6	1.066		
III	4	1.036	5	1.053	4	0.966		
Total	14	1.062	16	1.071	15	1.050	45	1.066
$\bar{S}_{y \cdot x}$		0.030		0.028		0.028		
σ_k		0.011		0.012		0.020		
P		0.0002		0.0002		0.0271		
B. Phospholipid versus Total Lipid								
I	4	0.624	5	0.479	5	0.149		
II	6	0.707	6	0.553	6	0.569		
III	4	0.733	5	0.437	4	0.642		
Total	14	0.704	16	0.559	15	0.648	45	0.628
$\bar{S}_{y \cdot x}$		0.122		0.225		0.183		
σ_k		0.046		0.094		0.130		
P		0.0002		0.0004		0.0182		
C. Total Cholesterol versus Total Lipid*								
I	4	0.707	5	0.325	5	−0.411		
II	5	0.740	5	0.698	5	0.694		
III	3	0.916	4	0.842	3	1.133		
Total	12	0.824	14	0.713	13	0.716	39	0.752
$\bar{S}_{y \cdot x}$		0.136		0.161		0.183		
σ_k		0.056		0.070		0.132		
P		0.0108		0.0014		0.0558		
D. Total Fatty Acids versus Total Lipid								
I	4	1.028	5	1.027	5	1.063		
II	6	1.021	6	1.025	6	1.019		
III	4	1.010	5	1.014	4	0.970		
Total	14	1.017	16	1.020	15	1.016	45	1.019
$\bar{S}_{y \cdot x}$		0.010		0.009		0.011		
σ_k		0.004		0.004		0.008		
P		0.0004		0.0002		0.0533		

* The omission of data of Series II at 150 days and of Series III at 175 days is explained in the text.

two lower temperatures and almost so at 30°. Direct comparisons indicate no significant differences between the *k*-values for the three temperatures (Table 6, E). The analysis of variance supports this conclusion (Table 7, E), for whatever variation is present is seen to reside principally among experiments.

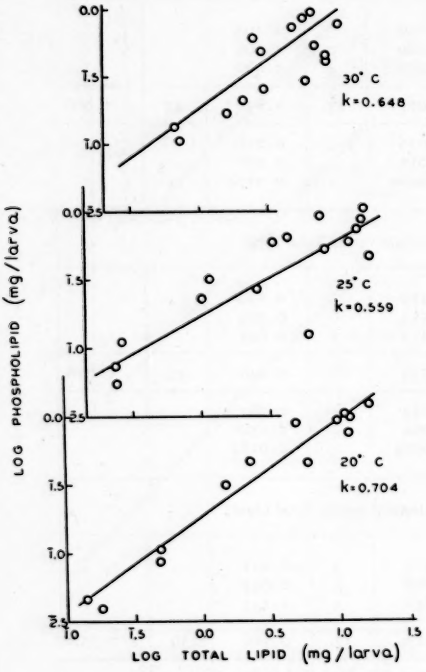

FIG. 6.—Double-log plot of phospholipid against total lipid at various temperatures.

DISCUSSION

The data presented demonstrate that, among *Tenebrio* larvae, there is an appreciable degree of variation in growth, even when the organisms are grown under identical conditions of temperature and humidity. These findings with respect to variability are consistent with the experience of other investigators working with this animal, e.g., Cotton (1927). As is evident from Table 2, not only is there variation from culture to culture, but considerable differences are also present among the individuals within a culture. It is doubtful that lack of food or the accumulation of waste products was responsible for the variation, since the bran in which the larvae developed was changed frequently enough to provide an ample supply of fresh medium.

In addition to the general tendency toward variability, there seems to be

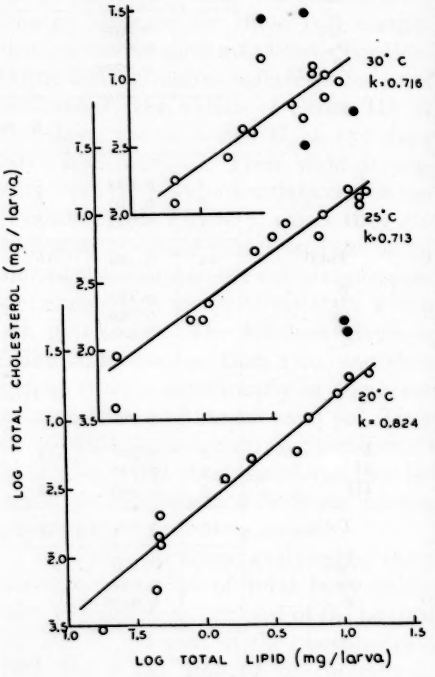

FIG. 7.—Double-log plot of total cholesterol against total lipid at various temperatures.

some relation between temperature and the degree of variation, at least over the range studied. The mean coefficients of variation for weights within a culture (Table 2) show a progressive increase with higher temperature levels. This situation is in contrast with that of the rela-

tive growth constants of the lipid components, for not only is the extent of variation of these of the same order of magnitude from temperature to temperature within each category of lipid (Tables 5 and 8), but there is at least an equal degree of consistency between temperatures as there is between experiments run at each temperature (Table 7).

The differences among the growth curves of the larvae at the three temperatures studied (Fig. 1) probably can be accounted for by certain well-established principles: (1) Except at the extremes of the tolerated temperature range, poikilothermic organisms tend to develop more rapidly at higher temperatures. This needs no elaboration, having been well established for insects (Uvarov, 1931) as well as for other organisms. For *T. molitor*, in particular, Lengerken (1925) reported that the length of larval life was increased by many months and that supernumerary molts occurred when these insects were kept in a cool cellar. Singh-Pruthi (1924) also showed that many more molts occur in these forms at 23° and 26°.5 than at 29°.5 C. (2) Many animals, although they develop more slowly, tend to reach a larger size when they are raised at lower temperatures. Titschak (1925) showed that larger and heavier clothes moths (*Tineola biselliella*) were produced at lower temperatures. He accounted for these results by the increase in the length of larval life and in the total amount of food ingested, by the greater percentage of food absorbed, and by the greater utilization of food for the building of body tissues at colder temperatures. Uvarov (1931) cites other instances of larger size at lower temperatures in insects, while Hesse, Allee, and Schmidt (1937) refer to many similar instances among marine organisms. (3) Many insects living on stored products grow faster when the relative humidity is higher (Ahmad, 1936; Fraenkel and Blewett, 1943). In a further study in 1944 the latter authors demonstrated that *Tribolium confusum*, *Ephestia kuhniella*, and *Dermestes vulpinus* ate more food under drier conditions to produce a given unit of body weight, while the moisture content of the pupae remained practically constant. Under these circumstances the length of the larval period increased, the larvae grew more slowly, and the weight of the pupae decreased. In this connection, Evans and Goodliffe (1939) showed that the feeding larvae of *Tenebrio* obtain practically no water from ingested bran, but they calculated that metabolic water is produced from the combustion of carbohydrates. Since, in the present experiments, the highest value for relative humidity was associated with the lowest temperature, it is

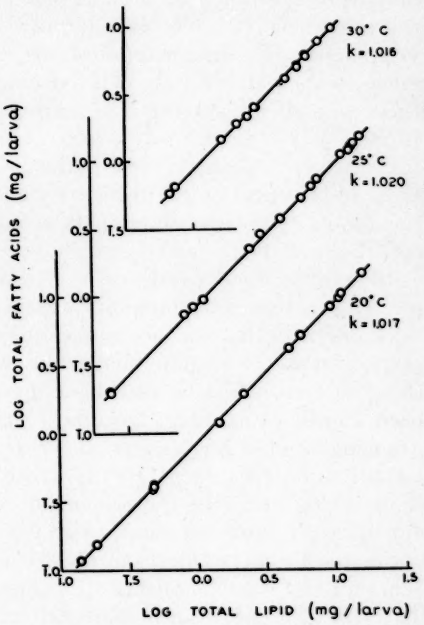

FIG. 8.—Double-log plot of total fatty acids against total lipid at various temperatures.

not surprising that this curve of larval growth was heightened and the duration of larval life shortened by virtue of the effect of moisture. This conclusion is further strengthened by the consistent picture obtained when the present data are plotted together with those of Menusan (1936) in Figure 2.

Another effect of high temperature may be of additional value in explaining the growth curves. The late larval development and the metamorphosis of meal worms is known to be delayed when these animals are subjected to temperatures of 30° C. (Singh-Pruthi, 1924). This finding may explain the drop, after 200 days, in the curve at 30° in Figure 1 and the almost complete failure of these cultures to produce pupae.

In spite of the dependence of growth on temperature and humidity, earlier work indicates that the moisture content of the larvae is largely unaffected by these factors. Meal worms have long been known to maintain an almost unchanging ratio of body water under dry conditions (Berger, 1907). Buxton (1930) showed that at 23° C. the proportion of dry matter to water is constant at various humidities in fasting *Tenebrio* larvae. Whether this is accomplished by changing rates of metabolism at different humidities (Buxton) or by altered rates of evaporation (Mellanby, 1932, 1934, 1936), the important point here is that meal worms are capable of maintaining a constant moisture content under a variety of external circumstances (except under hot, moist conditions). Mellanby (1932) suggested that normal meal worms lose water at a rate proportional to the saturation deficit when the relative humidity is less than 88 per cent. He pointed out that, because of their marked ability to conserve water, Tenebrionid beetles are adapted to live in hot, dry air or in cooler air at various humidities. In view of the fact that many physiological processes among insects are dependent upon the relative humidity, while many others are correlated instead with the saturation deficiency (Ludwig, 1945), it was considered to be sufficient in the present work to maintain the cultures at fairly constant humidity levels in the middle of the tolerated moisture range. The differences in the resulting curves of growth fortified the validity of the conclusions concerning the heterauxetic constants.

Previous studies of meal worm lipids have consisted largely in charting the changes during pupation. Becker (1934) found that larval and pupal fats were practically indistinguishable qualitatively and that young adult fat was very similar, except for an increase in nonsaponifiable matter (4.4 per cent against 0.93 for pupae and 1.56 for larvae). This trend became even more marked in old adults, in which the unsaponifiable matter reached 6.3 per cent. In addition to these qualitative changes, he found that the percentage of fat declined with metamorphosis, being 12–14 per cent in larvae, 8.9 in pupae, 8.1 in young, and 4.6 in old adults. On the other hand, Evans (1934) found that the amount of unsaponifiable material, as well as the amount of fatty acids, remained constant during the transformation from larva to adult.

The present study was concerned with an earlier phase of meal worm development. Here, in the long larval period, definite trends in the changing lipid composition appeared. Total lipid was found to become an increasing proportion of body weight, ranging from 8–9 per cent of the fresh weight at 50 days to 12–17 per cent at pupation or at 200 days. The latter range is consistent with Becker's findings. It is not surprising that such an increase should occur, for the larval pe-

riod is commonly thought to be a specialized portion of the life-cycle devoted to food-gathering and deposition of nutritional reserves. In insects this larval role is emphasized by the presence of a storage organ, the fat body, which, Wigglesworth (1939) points out, is most conspicuous in the larvae of holometabolous forms. The fat content of certain insects, silkworms in particular, reaches rather high values (Uvarov, 1928). Rudolfs (1926) followed the fat content in the tent caterpillar throughout development and found that during the period from the freshly hatched larva to pupation the ether-soluble extract in percentage of dry weight rose from about 1 to 29 per cent. Kemnitz (1916), with the botfly, *Gastrophilus equi*, found a steady increase of ether extractives from 3.54 to 26 per cent on a dry-weight basis (corresponding to 0.8–9.0 per cent of fresh weight) in larvae ranging in fresh weight from 0.2 to 0.515 gm. In the flesh fly, *Lucilia sericata*, total ether extractables were found by Yuill and Craig (1937) to increase from 6 per cent in young larvae to 30 per cent in fully grown ones. Melampy *et al.* (1940) reported increases in the total lipid in the larvae of queen and worker honeybees, with a maximum at or shortly before pupation. Similar results had been obtained by Straus (1911) for worker and drone bees.

What gives these data on insects larger significance is the fact that they are consistent with trends widely found throughout the animal kingdom. An increase in the percentage of fat with growth has been found in chick embryos (Murray, 1926; Romanoff, 1932, and others cited by Needham, 1931), in rabbit fetuses (Fehling, 1877), and in human fetuses, (Fehling, 1877; Aron, 1913; Iob and Swanson, 1934). A slight rise in the absolute amount of fat as well as its percentage of the dry weight was found by Gortner (1914) and by McClendon (1915) in growing salamander eggs, while Needham (1931) cites the work of Burdach in 1853, who found an increase in neutral fat in the egg of the snail, *Lymnaea stagnalis*, during development. This rise does not represent a universal phenomenon, however, for Gortner (1945) found that in pig embryos the total lipid percentage of the wet weight remained fairly constant at about 1 per cent.

In addition, mammals show a striking increase in fat content with age in the postnatal period (Moulton, 1923). This generalization is supported by the work of Chanutin (1931) and more recently of Williams *et al.* (1945) on rats. The importance of this trend is attested to by the long-standing practice of reducing data on chemical composition of tissues to a fat-free, as well as a moisture-free, basis (Aron, 1913). Parallel with the increase in total fat is the well-established decrease in the relative water content (Moulton, 1923; Needham, 1942).

The increase in the relative fat content which occurs during growth is not a simple measure of the storage of reserve materials. After the work of Mayer and Schaffer and of Terroine it has been recognized that fatty substances in tissues can be divided into two great categories: the *élément constant*, consisting of the phospholipids (especially of lecithin) and of cholesterol, and the *élément variable*, comprising the neutral fats (Bloor, 1945). The latter represents the storage deposits, while the former group of substances is essential to and characterizes tissue structures and activities. The lipids of the meal worm show a generally increasing proportion of neutral fat, reaching 90–94 per cent of the total lipid by 200 days, reflecting the accumulation of reserves preceding pupation. This trend is mirrored by the generally decreasing percentage of essential lipids

(phospholipid and cholesterol). Young rats show similar trends in the percentage lipid distribution, with neutral fat rising from 55 per cent at birth to 87 per cent at 70 days, while there is a concomitant downward trend for essential lipids (Williams *et al.*, 1945).

In terms of its relation to total fresh weight, the phospholipid of meal worm larvae was seen to constitute an almost constant proportion, declining slowly from 1.5–2.2 per cent at 50 days to 0.7–1.0 per cent at 200 days. Calculations from Teissier's data (1931) show a similar steady decline in lipid phosphorus from 0.089 per cent in 10-mg. larvae to 0.053 per cent in 205-mg. larvae, roughly corresponding to from 2.34 down to 1.39 per cent in values for phospholipid. Needham (1942) summarized earlier work on phosphatides in the chick by stating that they are built into the embryo bradyauxetically with the relative growth constant at about 0.75. This value agrees fairly well with that found for meal worms (Table 8, B). In mammals the picture is confused by the rapidly changing water content immediately after birth. In terms of dry weight, however, both Sinclair (1930) and Williams *et al.* (1945) found decreasing percentages of phospholipid in the postnatal development of the rat. Boyd (1935), on the other hand, found an increase in the wet-weight concentration of phospholipid in fetal rabbits with rates presumably varying with the degree of physiological activity (i.e., growth of the tissues), while Gortner (1945) reported practically no change in fetal pigs during development.

The total cholesterol in meal worms at various ages was found to be quite constant in terms of percentage of wet weight. This is in agreement with the concept of the constancy of cholesterol levels in many tissues (Bloor, 1945). Unvarying levels were also found by Gortner in pig fetuses, although again Boyd found a substantial rise in the free cholesterol paralleling that of the phospholipids in fetal rabbits.

Needham (1942) pointed out that, when the magnitudes of various chemical components are plotted against the magnitude of the entire organism, the ratios obtained constitute a type of "chemical ground plan of animal growth." In terms of heterauxesis, certain of the relationships examined here for meal worm larvae show clear-cut results. The relative rate of increase of total lipid with respect to the total wet weight was found to be consistent with that found by Teissier and to be significantly tachyauxetic. The constants for neutral fat and for total fatty acids against total lipids similarly were found to be significantly greater than 1.00, while that for phospholipid against total lipid was found to be bradyauxetic. It is possible to express the relation of each of these lipid entities to the total wet weight by multiplying the heterauxetic constants of Table 8 by 1.149, which is the k-value for total lipid versus the entire organism. Similarly, these relationships can be expressed in terms of total dry weight of the larva by further dividing by 1.06, which is the value for k found by Teissier for the relation between dry weight and wet weight. The constants arrived at by these calculations, which are given in Table 9, represent a type of ground plan of lipid growth in meal worm larvae expressed in terms of the concepts of chemical heterauxesis.

Interesting results are obtained when data from Series II at 225 and 250 days at 25° and at 225, 250, and 275 days at 30° C. are combined with the material up to 200 days already presented. When these additional points are included, the k-value for total lipid verses the entire organism at 25° is 1.210 and at 30° it is 1.054, in contrast to the values of 1.202

and 1.165, respectively, given in Table 5 for the coefficients of all the data at each temperature. For all the temperatures together, the regression coefficient becomes 1.135, in comparison with 1.149, which is obtained when the data are restricted to 200 days. The significance of these calculations lies in their demonstration that, when the total weight of the organisms declined, the total lipid decreased proportionately, so that a relatively fixed pattern was maintained.

The question of the flexibility or deformability of such a pattern under externally imposed stresses is part of the larger problem of the dissociability of ontogenetic mechanisms in general. Needham (1942) has shown that in many respects the various processes of growth, development, and metabolism can occur independently. Growth and differentiation, nuclear and cell division, histogenesis and organogenesis, and growth and metabolism have been shown to be differently related to one another, depending upon the conditions under which they are examined. Whether temperature has any effect in disengaging any of these mechanisms reduces in large part to the role of mere changes in the velocity at which these events occur.

Various aspects of lipid heterauxesis in meal worms have been seen here, by various analyses, to be independent of temperature. Not only did the relative growth constants, by and large, fail to show any significant differences on direct comparison, but more elaborate evaluation by analysis of variance techniques showed that changes in temperature were likely to be less important than variation from experiment to experiment. This finding is consistent with the data collected by Needham, which point to the conclusion that chemical composition is related to size and weight rather than to age.

The meal worms in the present study were subjected not only to different temperatures but at the same time to a differing set of humidities and vapor-pressure deficits. This combination of circumstances and the growth curves which they produced served to emphasize the subtle effects which result from simple ecological factors operating together. That the differential growth ratios remained constant in spite of the differences in temperature and degree of moisture in the environment serves to underline the conclusion that the magnitude of the organism, rather than the curve of its development, is the independent variable of chemical heterauxesis.

TABLE 9

HETERAUXETIC CONSTANTS IN TERMS OF WET WEIGHT AND DRY WEIGHT

Component	Total Lipid	Wet Weight	Dry Weight
Dry weight	.....	1.06*	.....
Total lipid	.....	1.149	1.084
Neutral fat	1.066	1.224	1.154
Total fatty acids	1.019	1.171	1.104
Phospholipid	0.628	0.722	0.681
Cholesterol	0.752	0.863	0.814

* From Teissier, 1931.

SUMMARY

1. Larvae of the meal worm (*T. molitor* L.) were raised at 20°, 25°, and 30° C. The cultures were sampled periodically and were analyzed for total fatty acids. cholesterol, and phospholipids. Total lipid and neutral fat were calculated from these determinations.

2. The dependence of rate of growth and duration of larval life on humidity, as well as on temperature, was confirmed.

3. Total lipid, neutral fat, and total fatty acids became increasing proportions of the wet weight as larval growth progressed. Phospholipid declined very slowly, while total cholesterol remained

practically constant in relation to wet weight.

4. Neutral fat increased, while phospholipid decreased in relation to total lipid as the larvae developed; total cholesterol decreased very slowly.

5. Examination of the data in terms of chemical heterauxesis revealed that total lipid is significantly tachyauxetic in relation to wet weight. With respect to total lipid, neutral fat and total fatty acids are tachyauxetic, while phospholipid and total cholesterol are bradyauxetic; these relationships are statistically significant.

6. At the three temperatures studied, the heterauxetic constants for each component do not differ significantly either by direct comparison or by analysis of variance.

7. The trends in lipid composition during the development of *Tenebrio* larvae are consistent with those known to exist in other insect larvae and in vertebrate embryos.

8. The heterauxetic constants represent a type of chemical ground plan of larval growth. This pattern appears to be unchanged by varying external conditions of temperature and humidity.

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A STUDY OF THE QUANTITATIVE EFFECTS OF ETHYNYL TESTOSTERONE UPON THE SWORD AND GONOPODIUM OF XIPHOPHORUS HELLERII[1]

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IT HAS been established that the male secondary sex characters of immature male and female Poeciliid fish can be induced to develop by the administration of androgenic hormone. Immature female fish, which are believed to have genetic male secondary sex characters, develop masculine characters upon the administration of androgenic hormone. Witschi and Crown (1937) have found that testosterone propionate administered to pregnant *Xiphophorus hellerii* causes absorption and resorption of young in a few days. The ovaries, upon histological examination, resembled testes, but there was no evidence of spermatogenesis. They claim that the females assumed the male coloration, gonopod, and complete development of the sword. Regnier (1938) applied testosterone propionate to immature male and female *X. hellerii*. Male secondary sex characters, including a well-developed sword, consistently developed. Eversole (1939) obtained in female *Lebistes reticulatus*, injected with testosterone propionate, atypical male characters, such as the suppression of body growth, atypical gonopodial development, and black spots behind the anal fin. The gravid spot, which is characteristic of females, was not suppressed. In 1941, Eversole, using ethynyl testosterone, induced male characters in females. The gravid spot was suppressed in immature females but could not be suppressed in mature females.

These experiments established the fact that treatment of normal male and female immature fish with high concentrations of male sex hormone produces masculinizing effects. The next step was an investigation of the effects not only of low concentrations of androgenic hormone on gonopodial development but of the optimal levels of low hormone concentrations at which certain stages of gonopodial development occurred. Likewise, investigations were made to see whether smaller amounts of testicular tissue would be sufficient to produce a normal gonopodium in the male.

From his studies on female *Gambusia affinis* treated with ethynyl testosterone, Turner (1941) concluded that in low concentrations there was a thickening of ray 3 and a stimulation of growth of rays 3, 4, and 5. The 3, 4, 5-ray complex in female specimens treated with high concentrations was stimulated to develop into a precociously terminalized male gonopodium. St. Amant (1941) found in *G. affinis* that complete castration prevented metamorphosis of the anal fin into the gonopodium. Assuming that different levels of hormone concentration might induce certain levels of gonopodial development, he found that a piece of regenerating tissue 1/45 to 1/100 the size of the normal testis would maintain gonopodial development. Pieces of re-

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generating tissue, 1/173 that of normal testis, stimulated slight gonopodial growth. He concluded that small amounts of developing testes initiated gonopodial growth; higher levels of hormone concentration secreted by the testis completed anal-fin metamorphosis. Grobstein (1942) produced an atypical gonopodium by administering, intraperitoneally, testosterone propionate to normal females of *Platypoecilus maculatus*. Adult females, treated similarly with testosterone propionate, during regeneration of the anal fin produced gonopodia more nearly like those of the normal male. Grobstein further found that the period of maximum sensitivity of the regenerating anal fin to androgenic hormone stimulation was the period approximately between 10 and 15 days of regeneration. Injections of low dosage, prior to this time, had produced but slight effect, while high dosage produced the atypical gonopodium. Turner (1942) sought the minimal dosage of ethynyl testosterone required to elicit response of differentiation areas in the anal fin of female *G. affinis*. He found that 1 mg. of ethynyl testosterone in 14,000,000 cc. of water induced the beginning of development of ray 3. The optimal concentration for growth and formation of all differentiation areas is 1 mg. of ethynyl testosterone in 2,000,000 cc. of water. Higher concentrations produce early differentiation and termination of growth. Cummings (1943), injecting *Mollienisia latipinna* with testosterone propionate, found that the interval between injections and the amount of dosage administered at each injection caused definite variations in the development of the gonopodium. However, Cummings discovered the minimal concentration of testosterone propionate which would just initiate development and the growth of the gonopodium in the 3, 4, 5-ray complex was 1/5,000 mg. per cubic centimeter at only 0.03-cc. dosage at 3-day intervals. Concentrations of 1/10,000 produced no effects.

The next step was to investigate the effect of androgenic hormones upon the secondary sex characters of such structures as the sword of *X. hellerii* and to correlate from such studies the development of the gonopodium with the sword at different levels of androgenic hormone. Therefore, an attempt was made to determine in immature males and females the amount of hormone necessary for gonopodial growth, gonopodial terminalization, and the amount of hormone necessary to promote the growth of the sword.

MATERIALS AND METHODS

Mature gravid female *X. hellerii* were bought from an aquarium supply-house in Chicago. From these specimens young were obtained. When hatched, the broods were placed in separate aquaria until ready for use. Young specimens ranged in length from 9 to 12 mm.

The fish, during experimentation, were placed in 1-gallon milk jars containing 3,000 cc. of ethynyl testosterone solutions in concentrations of 1 mg. of ethynyl testosterone in 20,000 cc. of water to 1 mg. in 3,000,000 cc. of water. Only one fish was placed in each jar. They were fed, daily, fish food consisting of dried liver, shrimp, egg, and milk. The fish were kept at room temperatures ranging from 20° to 30° C. Since, in these young fishes, it was impossible to distinguish the sex by external examination, it was necessary to kill the specimens and to preserve and examine the gonads at the end of the experiment in order to determine the sex.

Because complete development of the normal male takes approximately a year, four specimens in various stages of devel-

opment were studied in sequence. From these studies a composite picture was constructed of the growth of the gonopodium and the development of the sword. The specimens were as follows: One fish was 15 mm. long, one was 26 mm., one was 31 mm., and one mature specimen was 41 mm. Data were taken from the 15-mm. specimen over 105 days. The data taken from the older specimens extended over a 3-week period. These data were plotted and fitted together in sequence to give the curves as shown in Figure 1. The figures given for the normal development of the male specimen are only approximate.

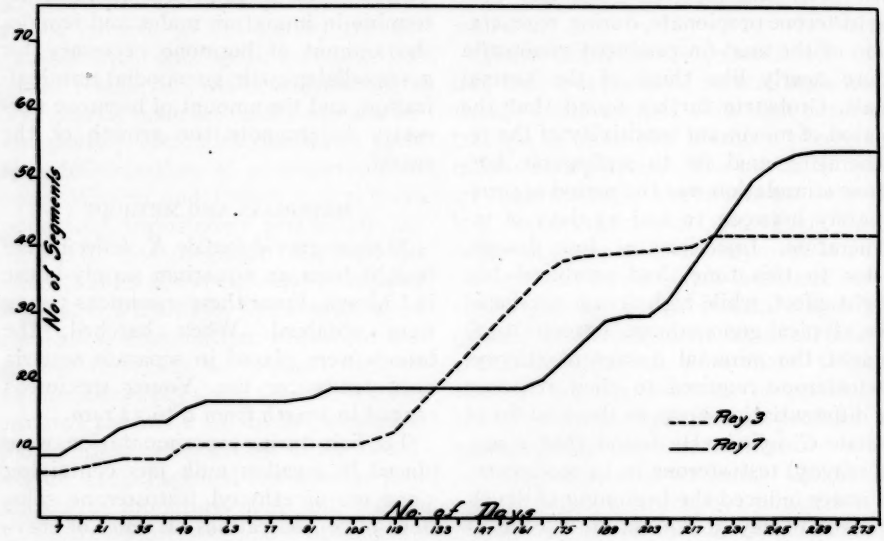

FIG. 1.—Growth curves of ray 3 of the gonopodium and ray 7 of the sword of the normal male of *X. hellerii* from the time of birth to maturity.

The growth of the gonopodium of *X. hellerii* was checked by determining weekly increases in the number of segments in ray 3 of the gonopodium. No attempt was made to observe the development of areas of differentiation of the gonopodium during these experiments. Because the sword develops from the ventral half of the caudal fin in the male, weekly checks were made of the increase in the number of segments of ray 7 in the ventral half of this fin.

Specimens were arranged in two series on the basis of sex. Series I*a* consisted of young female fish, 9–12 mm. in length when first placed in the hormone solution. Series I*b* consisted of older female fish, 14–16 mm. in length when placed in the hormone solution. The young females were placed in ethynyl testosterone solutions of 1 mg. in 40,000 cc., 1 mg. in 100,000 cc., and 1 mg. in 1,000,000 cc. of water. The older females were placed in ethynyl testosterone solutions of 1 mg. in 1,000,000 cc., 1 mg. in 2,000,000 cc., and 1 mg. in 3,000,000 cc. of water.

Series II consisted of male fish. Series II*a* comprised young male fish, 9–12 mm. in length when first placed in the hormone solution. Series II*b* consisted of older males, 16–19 mm. long. The young males were placed in ethynyl testos-

terone solutions of 1 mg. in 20,000 cc., 1 mg. in 1,000,000 cc., and 1 mg. in 3,000,000 cc. of water. The older males were placed in ethynyl testosterone solutions of 1 mg. in 1,000,000 cc., 1 mg. in 2,000,000 cc., and 1 mg. in 3,000,000 cc. of water.

The action of synthetic androgenic hormone on *X. hellerii* females in which the effects of testicular hormone was lacking was studied in Series I, while the additional effect of endogenous testicular hormone to synthetic hormone in the male fish was studied in Series II.

The length of the body was measured, by dividers, in millimeters from the tip of the snout to the base of the caudal fin (the standard length).

DEVELOPMENT OF THE NORMAL MALE

Figure 1 shows how slowly, at first, the secondary sex characters develop. Up to 105 days after birth the gonopodium shows little or no development. Ray 3 of this organ increased from 7 to 11 segments; but, during the period from 105 to 189 days, ray 3 grew from 11 to 40 segments. In the following 21 days (an overall total of 210 days) ray 3 added 2 segments. The sword, during a 161-day period, developed slightly. Ray 7 increased from 9 to 19 segments, but from 161 to 280 days, this ray developed an additional 36 segments.

EXPERIMENTAL RESULTS

SERIES I*a*. YOUNG FEMALES TREATED WITH ETHYNYL TESTOSTERONE (FIG. 2)

In specimen 1, 12 mm. long, treated with 1 mg. of ethynyl testosterone in 40,000 cc. of water, the gonopodial growth, as indicated by ray 3, was completed in 28 days. The segment number increased from 3 to 35 during this time. Ray 7 of the sword, however, showed a steady growth in segments. From the beginning of the experiment to termination of gonopodial growth, ray 7 had added 26 segments. At the time of death, in 77 days, the segment number of ray 7 increased from 8 to 78. The sword was 19 mm. long.

In specimen 2, treated with 1 mg. of ethynyl testosterone in 100,000 cc. of water, the curve of ray 3 indicates a fairly rapid development of the gonopodium during the first 42 days; then it levels off, indicating very slow growth during the following 28 days. In 70 days the ray had increased from 6 to 34 segments. Ray 7 of the caudal fin showed steady but slow growth, as compared with specimen 1. From the beginning of the experiment until terminalization of growth of the gonopodium, ray 7 had developed from 8 to 22 segments. At the end of the experiment, 126 days, ray 7 had 30 segments. The sword was 10 mm. in length.

Specimen 3, treated with 1 mg. of ethynyl testosterone in 1,000,000 cc. of water, showed a gradual completion of the gonopodium. Development was complete in 105 days. The segments of ray 3 of the gonopodium increased from 3 to 33 in this time. There was no development of the sword; ray 7 grew from 7 to 17 segments.

SERIES I*b*. OLDER FEMALES TREATED WITH ETHYNYL TESTOSTERONE (FIG. 3)

In 1 mg. of ethynyl testosterone in 1,000,000 cc. of water the gonopodium of specimen 4 took 84 days to complete its development. Ray 3 of the gonopodium increased from 7 to 41 segments in this 84-day period. At the completion of gonopodial growth, ray 7 of the caudal fin had expanded from 11 to 28 segments. At the end of the experiment, 105 days, the sword was 14 mm. in length. Ray 7 had 30 segments.

In 1 mg. of ethynyl testosterone in 2,000,000 cc. of water the gonopodium of specimen 5 had completed its growth in

105 days. At the time of gonopodial growth termination, ray 3 had increased from 7 to 28 segments. But in the 105-day period the sword was only feebly developed. The total number of segments in ray 7 was 17.

Specimen 6, placed in 1 mg. of ethynyl testosterone in 3,000,000 cc. of water, died in 84 days. The gonopodium showed but slight development in this period. Ray 3 had grown from 7 to 11 segments. The sword showed a slight development. Ray 7 of the sword had increased from 11 to 24 segments.

In this series of young and older females the effect is only of synthetic androgenic hormone uncomplicated by androgenic hormone from the testis. Nevertheless, synthetic androgenic hormone was capable of precociously stimulating the anal fin to develop into the gonopodium and terminate its growth. The ventral half of the caudal fin grows rapidly into an elongate structure in high concentrations of ethynyl testosterone, but more slowly in lower concentrations of the hormone. There was no evidence of growth terminalization of the sword.

The results of the experiments on females in this series shows further that the gonopodium is much more sensitive to ethynyl testosterone stimulation than is

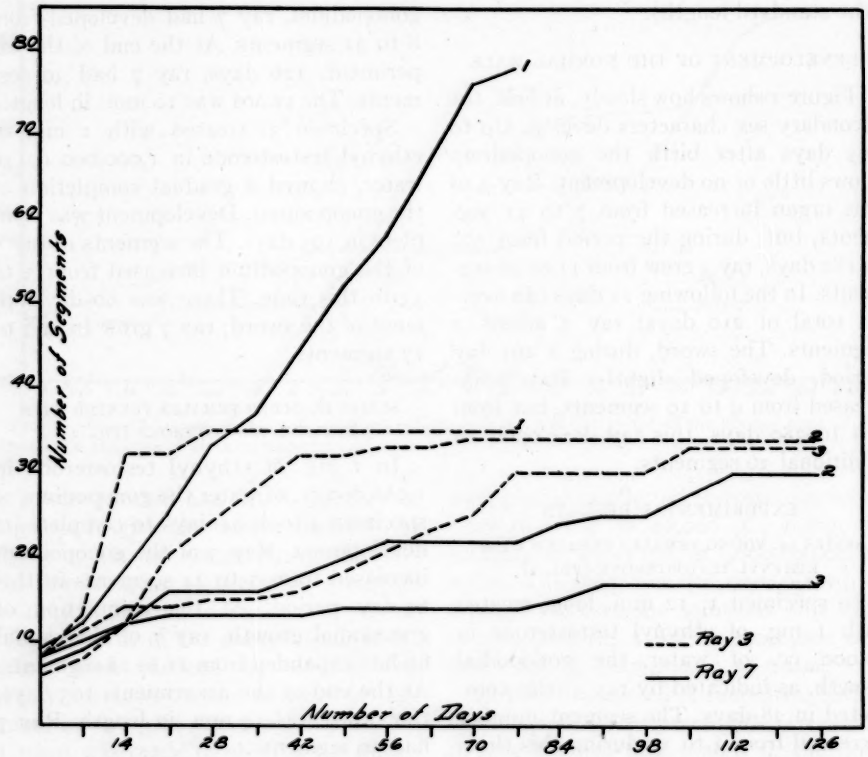

FIG. 2.—Young females. Number of segments added to ray 3 of the gonopodium and ray 7 of the sword of *X. hellerii* when treated with different concentrations of ethynyl testosterone. Specimens are indicated by numbers. Concentrations are as follows: *1*, 1 mg/40,000 cc; *2*, 1 mg/100,000 cc; *3*, 1 mg/1,000,000 cc.

the sword. The sword starts to grow later than the gonopodium and continues to develop after the growth of the gonopodium has ended. Concentrations which are sufficient to terminate growth in the gonopodium are insufficient to promote maximal growth of the sword. Thus in this series, specimen 5, treated with 1 mg. of ethynyl testosterone in 2,000,000 cc. of water, completed its gonopodial growth in 105 days. The growth of the segmentation and elongation. In 21 days, at the time of growth termination of the gonopodium, ray 7 had increased from 10 to 31 segments. Growth of ray 7 continued steadily until, on the 77th day, there was a total of 77 segments. At this point growth of the sword ceased. The length of the sword, at the end of the experiment—84 days—was 17 mm.

Specimen 8, treated with 1 mg. of ethynyl testosterone in 1,000,000 cc. of

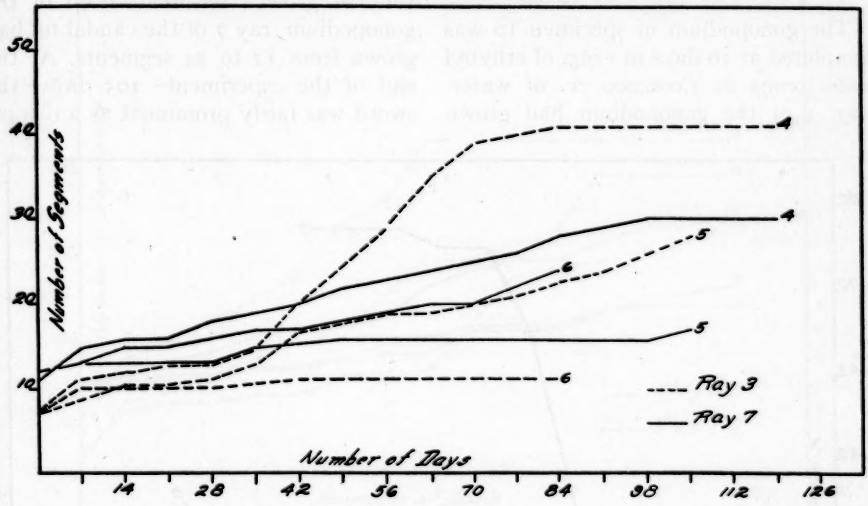

FIG. 3.—Older females. Number of segments added to ray 3 of the gonopodium and ray 7 of the sword of *X. hellerii*, when treated with different concentrations of ethynyl testosterone. Specimens are indicated by numbers. Concentrations are as follows: *4*, 1 mg/1,000,000 cc; *5*, 1 mg/2,000,000 cc; *6*, 1 mg/3,000,000 cc.

sword, however, was very slight. It is apparent, then, that the tissue susceptibility of the gonopodium to the stimulating effects of ethynyl testosterone solutions is much greater than is that of the sword.

SERIES II*a*. YOUNG MALES TREATED WITH ETHYNYL TESTOSTERONE (FIG. 4)

In specimen 7, treated with 1 mg. of ethynyl testosterone in 20,000 cc. of water, the growth of the gonopodium was completed in 21 days. Ray 3 of the gonopodium grew from 7 to 30 segments. Ray 7 of the caudal fin showed rapid water, completed its gonopodial growth in 28 days. Ray 3, during this period, increased from 5 to 36 segments. Ray 7 of the caudal fin, on the other hand, showed a steady development in the number of segments. At the period of growth termination of the gonopodium, ray 7 had grown from 9 to 17 segments. In 119 days ray 7 had 41 segments. The sword was 15 mm. long at the end of the 119 days.

Specimen 9, treated with 1 mg. of ethynyl testosterone in 3,000,000 cc. of water, showed slight gonopodial growth. In 63 days the number of segments had

increased from 5 to 11, but from 67 to 105 days there was a decided segment development. On the 105th day the number of segments in ray 3 was 45. Ray 7 of the sword, on the other hand, showed very slight effect of hormone stimulation. In 105 days the ray increased from 7 to 23 segments. The sword was 8 mm. long at the end of the 105-day period.

SERIES II*b*. OLDER MALES TREATED WITH ETHYNYL TESTOSTERONE (FIG. 5)

The gonopodium of specimen 10 was completed at 56 days in 1 mg. of ethynyl testosterone in 1,000,000 cc. of water. Ray 3 of the gonopodium had grown from 9 to 44 segments. At the completion of gonopodial growth, ray 7 of the caudal fin had increased from 15 to 27 segments. At the end of the experiment—105 days—the sword was 14 mm. long, and ray 7 had increased to 35 segments.

Specimen 11, in 1 mg. of ethynyl testosterone in 2,000,000 cc. of water, completed its gonopodial growth in 84 days, when ray 3 of the gonopodium had increased from 6 to 40 segments. At the time of growth terminalization of the gonopodium, ray 7 of the caudal fin had grown from 12 to 24 segments. At the end of the experiment—105 days—the sword was fairly prominent as a distant

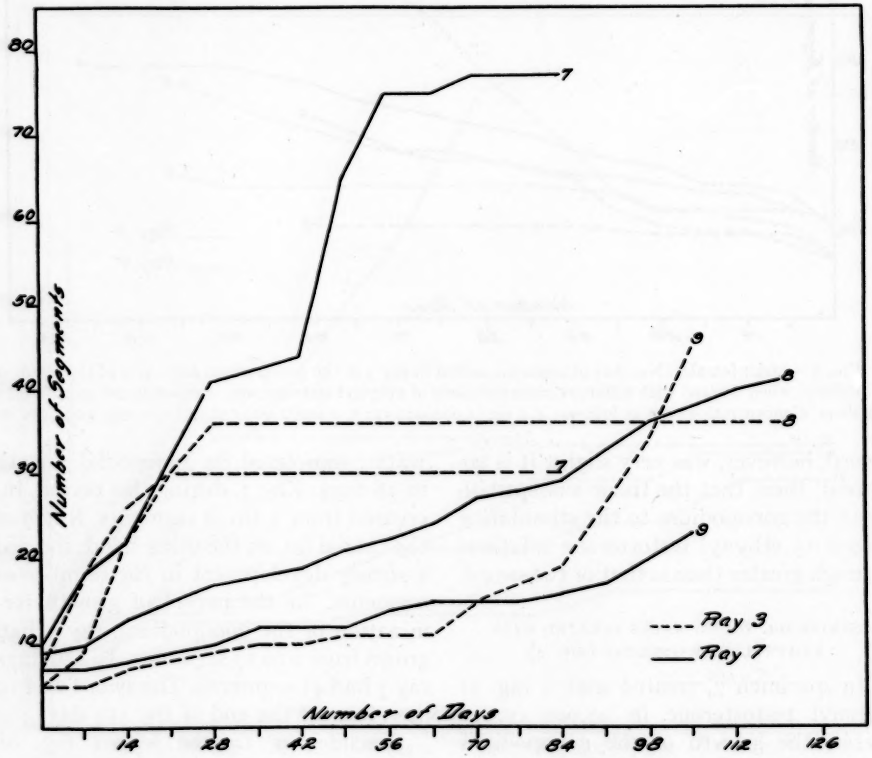

FIG. 4.—Young males. Number of segments added to ray 3 of the gonopodium and ray 7 of the sword of *X. hellerii*, when treated with different concentrations of ethynyl testosterone. Specimens are indicated by numbers. Concentrations are as follows: *7*, 1 mg/20,000 cc; *8*, 1 mg/100,000 cc; *9*, 1 mg/3,000,000 cc.

projection from the ventral side of the caudal fin and was 10 mm. long. Ray 7 in the 105-day period developed 27 segments.

The gonopodium of specimen 12 in 1 mg. of ethynyl testosterone in 3,000,000 cc. of water had terminated in 105 days, at which point ray 3 had increased from 7 to 34 segments and the sword showed slight development. In this 105-day period, ray 7 of the sword had grown from 13 to 25 segments.

Again, as in Series I, the experiments in this group show that the stronger the solution of ethynyl testosterone, the more quickly do the sword and the gonopodium develop. But, since the gonopodium is a highly differentiated structure, its growth is concluded much sooner than is that of the sword, which, being a relatively undifferentiated structure, continues to develop long after the gonopodium has completed its growth. In only the very strong solutions of ethynyl testosterone—1 mg. in 20,000 cc. of water—does growth termination of the sword take place. In weak solutions of ethynyl testosterone, on the other hand, as in 1 mg. in 3,000,000 cc. of water, although the gonopodium had completed its growth, the sword shows but slight effects of the hormone stimulation. It is again evident that the gonopodium is much more responsive to solutions of ethynyl testosterone than is the sword.

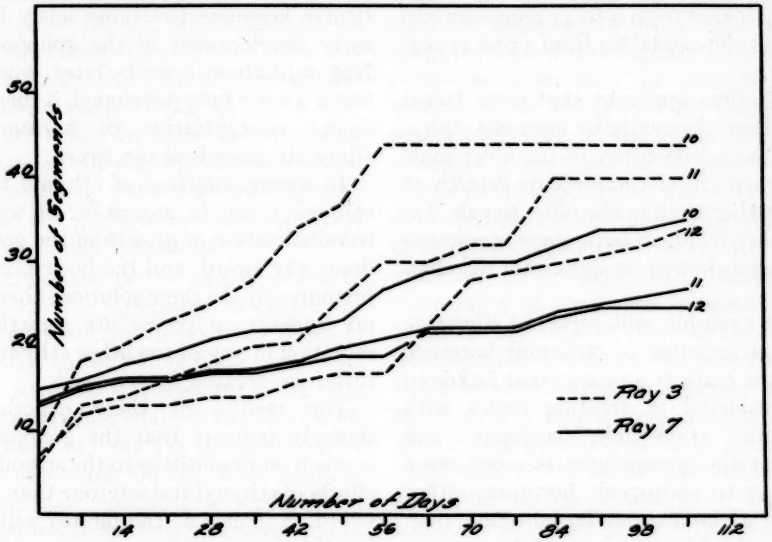

FIG. 5.—Older males. Number of segments added to ray 3 of the gonopodium and ray 7 of the sword of *X. hellerii*, when treated with different concentrations of ethynyl testosterone. Specimens are indicated by numbers. Concentrations are as follows: *10*, 1 mg/1,000,000 cc; *11*, 1 mg/2,000,000 cc; *12*, 1 mg/3,000,000 cc.

The release of androgenic hormone from the testis is an additional factor which must be considered in this series of males treated with ethynyl testosterone. In specimens treated with weaker solutions of ethynyl testosterone the influence of testicular hormone in the males is quite evident. For example, compare the older male—specimen 10—and the older female—specimen 4—both treated with 1 mg. of ethynyl testosterone in

1,000,000 cc. of water. In the older female (specimen 4) the gonopodium completed its development in 84 days. Ray 3 of the gonopodium increased from 7 to 41 segments in this period. Likewise, the sword in this 84-day period had increased from 11 to 28 segments. In the older male (specimen 10), treated with the same concentration of hormone, the gonopodial growth was completed in 56 days, during which time ray 3 of the gonopodium had increased from 9 to 44 segments and ray 7 of the caudal fin from 15 to 27 segments.

It is thus apparent that some factor other than the synthetic hormone stimulated the gonopodium of the older male (specimen 10) to complete its growth 28 days earlier than in the older female. On the other hand, in both these specimens the sword showed no significant development.

This example will serve to illustrate (1) that secretion of testicular hormone from the testis is an important factor to be considered in treating males with synthetic androgenic hormone and (2) that the gonopodium is much more sensitive to androgenic hormone, either with or without testicular hormone, than is the caudal fin. Similar conclusions can be drawn by studying the graphs of the other specimens treated with weaker solutions of ethynyl testosterone.

RECAPITULATION OF RESULTS

Comparing the results of the experiments in Series I and II with the normal development of the male (Fig. 1), we find that during the 119-day experimental period there is practically no development of the secondary sex characters in the normal male, although the gonopodium showed a segment increase in ray 3 of from 7 to 12 and the sword an increase in ray 7 of from 9 to 19.

Contrasting the development of the control male with that of the experimental specimens, it is found that not until 224 days (32 weeks) is development of the gonopodium completed and not until 161–68 days does the sword begin to develop. This would indicate that in the normally developing male the testis does not begin to secrete hormone in effective quantities until about 18 weeks after birth; and it would appear from the graphs that the initial output of the testicular hormone functions solely in the early development of the gonopodium. Not until about 6 weeks later, when the testis is more fully developed, is there sufficient concentration of hormone to stimulate growth of the sword.

In strong solutions of ethynyl testosterone—1 mg. in 20,000 cc. of water—terminalization of growth of the gonopodium, the sword, and the body occurred prematurely. In these solutions there was no evidence of premature growth termination in any of the other ethynyl testosterone–treated specimens.

The results of these experiments strongly indicate that the gonopodium is much more sensitive to the stimulating effects of ethynyl testosterone than is the sword. A glance at the figures will bear out this fact.

Since it is apparent that the gonopodium is much more sensitive to androgenic hormone than is the sword, we should expect the gonopodium to complete its growth in very weak solutions of hormone without any significant growth of the sword. On the other hand, we should expect the sword to begin its development in stronger solutions before terminalization of the gonopodium. Experiments in these two series indicate that the critical points for the beginning of the development of the gonopodium and the sword lie between 1 mg. of ethynyl testosterone in 2,000,000 cc. and 1 mg. in 3,000,000 cc. of water.

In the solution of 1 mg. of ethynyl testosterone in 3,000,000 cc. of water the gonopodium of the young male—specimen 9—was complete. Likewise, the older male—specimen 10—was complete and differentiated. Since both specimens were males, possibly sufficient ethynyl testosterone was present to stimulate the testis to produce added androgenic hormone, which would drive the gonopodium to completion. In specimen 6, probably an older female, which died in 84 days, the gonopodium did not show any significant development. The gonad was either an immature ovary or else a very immature testis. In either case the solution of 1 mg. of ethynyl testosterone in 3,000,000 cc. of water was not sufficient to drive the gonopodium to completion in the two male specimens.

A comparison of the 84-day period of the older fish—specimen 6—with the other two male specimens from Series II shows that in the young male—specimen 9—ray 3 of the gonopodium in 84 days had 19 segments, while in the older male—specimen 10—ray 3 of the gonopodium had 30 segments. On the other hand, in the older fish—specimen 6—ray 3 of the gonopodium in 84 days had 14 segments. The young male—specimen 9—had an immature testis, and the older fish—specimen 6—had either an immature ovary or an immature testis. At any rate, in both specimens the gonopodium had not started very far on its development. The older male—specimen 10—had a mature testis, so that in the 84-day period the gonopodium was well on its way to completion. It appears, then, that in the solution of 1 mg. of ethynyl testosterone in 3,000,000 cc. of water, without the aid of added hormone from the testis, the optimal concentration of ethynyl testosterone is just sufficient to drive the gonopodium to completion of its development.

The young male—specimen 9—and the older male—specimen 10 of Series II—when placed in 1 mg. of ethynyl testosterone in 3,000,000 cc. of water, showed significant development of the sword. Thus in the young male ray 7 of the sword increased from 7 to 23 segments, and in the older male ray 7 of the sword grew from 17 to 25 segments. It seems very likely that additional hormone from the testis was supplying the stimulus necessary to initiate the beginnings of sword development. There is considerable contrast between the development of the sword in these two males and that of the older female—specimen 5—in 1 mg. of ethynyl testosterone in 2,000,000 cc. of water. It is evident that the sword is in a lesser stage of development than in the two males. Nevertheless, its development is significant, since ray 7 of the sword increased from 12 to 17 segments. In this case the initial development of the sword was due entirely to the ethynyl testosterone alone, not from any added testicular hormone. These results indicate that the initial development of the sword in females and immature males occurs in 1 mg. in 2,000,000 cc. of water.

DISCUSSION

TISSUE AREAS SUSCEPTIBLE TO ANDROGENIC STIMULATION IN POECILIID FISHES

Experimental work with the androgens on the Poeciliid fishes shows that these fishes possess certain well-defined areas of high susceptibility. These tissues respond, when stimulated by ethynyl testosterone, for instance, in developing characteristic growth patterns. Such tissues in these fishes occur in the pelvic, pectoral, anal, caudal, and dorsal fins. These tissues, however, are not responsive to the same degree of intensity in all the species of the Poeciliidae, and, in fact, in some species only two or three of

these areas will respond to androgenic stimulation.

In *Gambusia* the susceptible tissue areas are the pectoral and anal fins. The other areas are not responsive. The pectoral fin reacts by a marked elongation in the male; the anal fin differentiates into the gonopodium. In *Lebistes* the susceptible tissue areas are the pelvic, anal, caudal, and dorsal fins. The pelvic fin elongates, the anal fin differentiates into the gonopodium, the caudal fin develops a short dorsal or ventral sword, or it may produce at the same time a double sword projecting from both the dorsal and ventral regions of the fin. The dorsal fin, rather than developing into the prominently high, rounded structure characteristic of the female, develops into a flat-topped, elongate structure. In *Xiphophorus* all the tissue areas, except that in the pectoral fin, respond to androgenic stimulation. The pelvic fins elongate, the anal fin develops into the gonopodium, the caudal fin produces a long ventral sword, and the dorsal fin tends to flatten out, as in *Lebistes*.

Hubbs in conversation has suggested that the various receptive tissues of the Poeciliid fishes possess different susceptibilities to androgenic hormone. This fact is entirely evident in the case of *Xiphophorus*, where the gonopodium possesses much greater sensitivity to ethynyl testosterone stimulation than does the sword. On the other hand, not only does each specimen possess certain areas of different susceptibilities, but also in each species of Poeciliidae similar areas of tissue vary. An example will make this clear. In females of *Gambusia*, Turner had found that the gonopodium is extremely sensitive to androgenic stimulation. He succeeded in inducing the first stage of gonopodial development in 1 mg. of ethynyl testosterone in 14,000,000 cc. of water. In 30 days, 1 mg. of ethynyl testosterone in 2,000,000 cc. of water were required to produce complete development of the gonopodium. In *Xiphophorus*, on the other hand, 105 days, in the same concentration of ethynyl testosterone, were needed to complete gonopodial development. The only explanation for such wide differences of response, in the examples cited above, is in the different genetic tissue susceptibilities of these two species.

THEORY OF ACTION OF ETHYNYL TESTOSTERONE ON THE GROWTH AND TERMINATION OF THE GONOPODIUM AND THE SWORD

Weiss (1939) advances the theory that the factors governing growth and differentiation are antagonistic. Thus the tendency in development is for growth merely to enlarge a certain tissue area by cell division; then differentiation sets in, and growth ceases. Since growth is the product of cell division, the protoplasm, as Weiss points out, must remain plastic, for it has no opportunity to utilize the cellular substance in specialized intracellular structure. During mitotic activity on the part of the cells, differentiation does occur; but, when the cells have completed their specialized intra-cellular organization, there is difficulty in returning to the plastic condition necessary for mitosis.

This concept of growth and differentiation as set forth by Weiss and the results of these experiments can be applied to the theory of action of ethynyl testosterone in gonopodial and sword development in *Xiphophorus*. The initial effect of ethynyl testosterone upon the receptive tissue of the sword and the gonopodium will be mitotic stimulation. In the receptive tissue the cells will undergo an abnormally large number of mitoses. Hence rapid growth of both the sword and the gonopodium will occur.

Xiphophorus has genetic factors for the

production, in the anal fin, of a highly differentiated structure, the gonopodium. The genetic factors in the receptor tissue cells of this structure will also, under stimulation of strong solutions of ethynyl testosterone, proceed to elaborate growth-stimulating substances which will act upon the developing gonopodium. These substances, elaborated at an abnormally high rate from these factors, possess the power of producing differentiation. They will then act upon the rapidly dividing cells and initiate differentiation of their cytoplasm, which, in turn, will retard or inhibit mitosis. Hence growth will cease at the onset of precocious differentiation, and the premature atypical gonopodium will develop.

It has been stated that the receptive tissue of the anal fin is genetically determined to produce a highly differentiated gonopodium. Now, if the receptive tissue of the gonopodium is subjected to an abnormally high concentration of ethynyl testosterone, as, for example, 1 mg. in 20,000 cc. of water, the normal well-regulated output of growth-stimulating substances will be greatly accelerated. Furthermore, if the growth-promoting substance is produced excessively, in all probability early exhaustion of this substance would likewise occur. Thus excessive production of the growth-promoting substance would tend to differentiate the anal fin into a gonopodium much earlier than in the normal condition. With precocious differentiation of the anal fin, mitosis will cease, and early completion of gonopodial growth will take place. But, furthermore, early exhaustion of the growth-promoting substance will also tend to end gonopodial growth because of lack of stimulation to mitosis.

The sword elongated continuously under the stimulus of ethynyl testosterone, and terminalization occurred only in the very high concentrations. This structure lacks the complicated differentiation pattern of the gonopodium, and its differentiation is expressed only in segmentation of the rays. These rays —6, 7, 8, 9, and 10—lying in the ventral half of the caudal fin, are the principal ones involved in the growth of the sword. Rays 7 and 8, which extend farther into the sword, are the most sensitive to ethynyl testosterone stimulation. In the caudal fin there is but one area of tissue differentiation in the tissue area at the base of these rays which is susceptible to ethynyl testosterone.

In high concentrations—1 mg. of ethynyl testosterone in 20,000 cc. of water—a sword was produced almost equal in length to the body. A large number of segments, in some cases slightly atypical, were developed. Terminalization was reached in 56 days, at a much later time than in the gonopodium. It is evident that this structure exhibits a minimum of differentiation. Hence growth, under ethynyl testosterone stimulation, was able to drive this structure into an abnormally long, trailing sword, without the antagonistic forces of differentiation coming into play. Nevertheless, in spite of the great length of the sword, terminalization was reached in 77 days in this particular example. It is likely that, under strong stimulation, the cells of the sword underwent rapid mitosis. Since there is but one area of differentiation, only one set of growth stimulators, elaborated by the genetic factors in the receptive tissue of the sword, acted upon the dividing cells. Since these growth stimulators are not substances for the production of complex structures as in the gonopodium, it is evident that mitoses will not cease. On the other hand, in extremely strong solutions premature enzymatic exhaustion, because of excessive ethynyl testosterone stimulation,

may occur and would result in eventual cessation of mitosis. Thus early growth terminalization of the sword would take place.

In weaker solutions premature exhaustion of the growth substance did not occur, with the result that mitosis continued under the stimulus of ethynyl testosterone. Thus early growth terminalization of the sword did not occur.

SUMMARY

1. Growth of the gonopodium and the sword of the normal male is slow. There is no significant development of the gonopodium until approximately 18 weeks after birth, and the sword does not begin to develop until approximately 24 weeks after birth.

2. In the normally developing male the testis does not secrete hormone in effective quantities for gonopodial development until approximately 18 weeks after birth. It would appear that after 18 weeks the developing testis is secreting hormone in amounts adequate for the early development of the gonopodium. Not until the testis is more fully developed, in approximately 24 weeks, is there sufficient hormone to stimulate growth of the sword.

3. Experiments upon female *Xiphophorus* indicate that the anal and caudal fins possess full genetic potentialities to develop into a structure typical of the male.

4. Females treated with ethynyl testosterone solutions showed that the higher the concentration of hormone, the earlier is the development of the gonopodium and the sword.

5. Males treated with ethynyl testosterone solutions show a similar, but more accelerated, growth response because of the added hormone supplied by the testis.

6. In strong solutions of ethynyl testosterone—1 mg. in 20,000 cc. of water—growth terminalization of the gonopodium and the sword takes place prematurely.

7. The susceptibility to the stimulating effects of ethynyl testosterone of the tissue of the gonopodium is much greater than that of the sword. Concentrations of ethynyl testosterone which are sufficient to complete growth of the gonopodium are not sufficient to promote maximal growth of the sword.

8. Genetic potentialities of similar tissues in each species of the Poeciliids varies. In females of *Gambusia*, kept in a concentration of 1 mg. of ethynyl testosterone in 2,000,000 cc. of water, 30 days were required for the complete development of the gonopodium. In *Xiphophorus*, in the same concentration of ethynyl testosterone, 105 days were required for complete gonopodial development.

9. A theory of ethynyl testosterone stimulation upon the sword and gonopodium of *Xiphophorus* is described in the discussion.

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SOCIAL FACTORS INFLUENCING THE HIERARCHIES OF SMALL FLOCKS OF THE DOMESTIC HEN: INTERACTIONS BETWEEN RESIDENT AND PART-TIME MEMBERS OF ORGANIZED FLOCKS[1]

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INTRODUCTION

DOMINANCE-subordination relationships form an aspect of social organization observed in many vertebrate animals from fish to man. Such relations have been summarized recently by Allee (1945, 1947) and by Collias (1944). In flocks of the domestic fowl this takes the form of a peck-order based upon peck-right. The dominant hen is at the top of the hierarchy and pecks all others without being pecked in return; a second hen pecks all except the first; and so on down to the omega individual at the bottom of the social order, which is pecked by all and which pecks none. Occasionally, exceptions to this straight-line hierarchy occur (cf. Schjelderup-Ebbe, 1913, 1922; Masure and Allee, 1934). The dominance-subordina-

tion patterns are apparently based on the ability of the hens to recognize one another individually and are influenced by the heredity, previous experience, physical condition, and environment of the individual animals concerned. These group organizations have been shown by Guhl and Allee (1944) to have advantages for the group as well as for the individuals of which it is composed and operate to increase mutual toleration within the group. On the other hand, organized flocks tend to oppose the presence of strangers and to fight to prevent them from joining the group. Social organizations of a similar character have been reported for flocks of birds in the wild or semi-wild condition by Odum (1941*a*) and Hamerstrom (1942) for chickadees, Jenkins (1944) for geese, and Emlen (1939) for valley quail.

The present work is an attempt to analyze some of the interactions between hens which are members of an organized flock and newcomers to the group, and between hens which are permanently present as compared with those which are with the group for only part of the time. To study these interactions, the existing hierarchy was first ascertained in each flock; then two hens were removed from one flock, introduced into a second group, and returned to the original hierarchy for short periods of time at regular intervals. This procedure was later repeated, so that some individuals became members of as many as five flocks simultaneously. In this way the interrelations between permanent members, newcomers, and part-time members could be analyzed. Further, the behavior was observed in flocks whose members were placed together for short periods, none of the members being permanent. Finally, two of the experimentally rotated hens were each implanted with a pellet of testosterone propionate to ascertain whether members present in as many as five flocks would rise in social rank as reported for hens permanently present (Allee, Collias, and Lutherman, 1939).

DEFINITIONS

It has been found convenient, both for clarity and for conciseness, to use various terms according to the following definitions:

1. The terms *flock* and *group* have been used interchangeably to indicate a number of hens which are housed in the same pen.

2. The *home flock* is the first or original flock in which an experimental hen is a member.

3. Members of a flock which are never moved to other flocks are termed *resident hens*.

4. The term *part-time member* has been used to indicate a hen which is not permanently present with the flock, i.e., one which is moved from one flock to another, spending part of its time in each. This term includes *absentee hens*, defined in paragraph 5; *visitors*, defined in paragraph 6; and *transients*, defined in paragraph 7.

5. When experimental hens are taken from the home flock and introduced into a second flock, they are considered *absentee* members of the home flock, since they spend only a limited amount of time there.

6. *Visitor hens* are those which are present in a flock other than the home flock for a short period of time, that is, up to 1 hour daily.

7. *Transients* are hens which are part-time members of the flocks with which they spend the night.

8. *The dominant* or *alpha* hen is the top-ranking member of the hierarchy. In most cases it can peck all the other hens without being pecked in return, although

there are some cases in which the generally dominant individual is involved in a triangle in which a subordinate hen pecks it. If the alpha bird never leaves the flock, it will be called the "resident dominant"; if it is a part-time member, the "visiting dominant," the "transient dominant," or the "absentee dominant," according to its appropriate classification.

9. The *beta, gamma, delta* hens, and so on, occupy the indicated lower positions in the hierarchy. The position of a hen is determined by the hens which it pecks. In case of a triangle, the status has been decided arbitrarily and is explained in a footnote in the tables.

10. In the text, *dominant* and *subordinate* refer to the relative social status and are used as purely objective terms. The alpha, beta, and gamma hens are considered relatively dominant individuals; the others are referred to as relatively subordinate in the small flocks used.

11. An *assimilated member of a flock* is a hen which feeds with the others, is not chased when on the floor, and does not apparently avoid the other members of the group continuously. An *unassimilated* hen obviously avoids the other hens, does not feed with the rest, and is chased whenever it comes near a member of the flock. Thus an assimilated hen is a true member of the social hierarchy and is allowed the privileges of flock membership, such as feeding or nesting, without interference from the group. An unassimilated bird is not treated as a member of the group by other hens; it is not accorded the privileges of flock membership without interference. A dominant newcomer can be considered assimilated as soon as it allows the other members of the flock to feed with it as freely as do most dominants in long-established groups. A relatively subordinate newcomer is said to be assimilated when it is allowed to feed and nest and walk around the pen as freely as resident members of the hierarchy with similar social status. The application of these criteria in determining whether a hen is assimilated, is, of course, somewhat subjective.

12. The proportion of the total number of pecks given and received by each member of the hierarchy has been computed and is used for purposes of comparison, in particular to determine whether part-time members delivered or received a higher proportion of pecks than did resident hens with a similar social status. These figures are influenced by both avoidance and aggression and therefore must be interpreted in the light of the observed behavior of the individual hens.

ANIMALS AND EQUIPMENT

Seven pens at the Whitman Laboratory were used for this work, four in a large poultry-house and three in a nearby greenhouse. Those in the henhouse are each 14.5 feet long, 4.5–5.0 feet wide, and 8 feet high. Each of the first three has two inner doors made of chicken wire, which, when closed, separate the rear third of the pen from the front area. Every henhouse pen has its own large south window and a small doorway, which leads to a separate porch for each pen, adjacent to the south side of the house. The porches have wire sides, bottoms, and tops and were used by the birds during the summer of 1945. The members of the different flocks could see one another through the porch sides, but the groups were not allowed to mingle. They could not see the other flocks when inside the poultry house (cf. Allee, Collias, and Lutherman, 1939). In the greenhouse there were three separate pens. Pen 5, in the center, was approximately 9 feet long and 5 feet wide. Pen 6 formed

an L around pen 5 to the right and the back and was separated from pen 5 only by 4×4-mesh, 22-gauge hardware cloth, so the two flocks could see each other readily. Pen 7, to the left of pen 5, was approximately 5 feet wide and 13 feet long and separated from pen 5 by heavy beaverboard.

Each of these pens was provided with four nests and raised roosts. In the henhouse there were also raised wooden platforms beneath the roosts. The cement floors of all were thickly covered with wood shavings, changed weekly. In the henhouse, daylight was supplemented by artificial light, regulated by a time clock to provide approximately sixteen hours of light daily. The temperature of the poultry-house was somewhat controlled by a thermostat and ranged in winter from 10° to 15° C., while in the summer it fluctuated with the weather, sometimes mounting as high as 34° C. The temperature of the greenhouse was higher in winter and slightly cooler in summer.

The individual observation pens into which certain experimental hens were rotated for part of the time were located in the greenhouse. These have been described elsewhere by Guhl and Allee (1944), who used them as isolation pens. They were each 4 feet long, 2.3 feet wide, and 2.5 feet high. Three sides were made of beaverboard, so that adjacent hens could not see one another, while the fourth side, the top, and the bottom were of chicken wire.

Sufficient mash food, oyster shell, grit, and charcoal were present in all pens continuously. Water was changed daily. Each flock was fed cod-liver oil mixed with mash three times weekly and a head of cabbage every seven days. Coarse grain scratch was present in a food hopper for one hour daily at observation time.

White Leghorn hens from an Indiana Farm Bureau stock which had been linebred since 1932[3] were used exclusively throughout the two years of observations. For identification, each hen was marked with colored leg bands. The four flocks of hens observed during the first year are called i, ii, iii, and iv, while the eight second-year flocks are designated by Arabic numerals.

During the first year of observations two flocks, i and ii, each had 6 hens received in the laboratory at the end of their first laying season in June, 1942. These birds had been used in somewhat similar dominance-subordination studies. Flock iii consisted of 9 new birds, received in the laboratory in October, 1943, when approximately one year old. The fourth group was composed of 5 one-year-old birds which were sent to the laboratory in February, 1944. Four hens in flock i died and were replaced by 3 new birds in February, and 3 birds from flock iii died but were not replaced. Flock ii received 2 new members and flock iv, 1. Thus a total of 32 hens was used during the first year.

Flock i occupied pen 1 in the henhouse until May 10, 1944, when it was moved to pen 5 in the greenhouse. Flock ii was in pen 5 until May 10, when it was moved to pen 1 of the henhouse. Flocks iii and iv were in greenhouse pens 6 and 7, respectively.

Throughout the second year the same four flocks were continued, and four additional groups were observed. These groups were numbered according to the pen in which they were housed, with the exception of the experimental flock, 8, discussed elsewhere. Thus former flocks i, ii, iii, and iv became 5, 1, 6, and 7, respectively. Flock 3, with 5 birds, and flock 8, with 4, were both former experi-

[3] All hens were furnished by Mr. Wayne Livengood of Terre Haute, Indiana.

mental groups from the June, 1942, shipment. Flocks 2 and 4 each had 7 hens received at the start of their first laying season in October, 1944. A total of 11 hens died during the course of the year, 1 each in flocks 2, 5, and 7; 2 in flocks 3 and 4; and 4 in flock 6. Two new residents were introduced into flock 3 and 1 into flock 4, so that a total of 48 hens was used during the second year.

PROCEDURE

The experimental work was carried out during two consecutive years, from October, 1943, to July, 1944, and from October, 1944, to September, 1945. The experimental procedure consisted in removing two hens from their home flock and introducing them into a second flock, where they then remained most of the time. They were then returned to the home flock for a short period either daily or on alternate days. In some cases this procedure was later repeated, and the partners were introduced into a third flock, where they now spent the greater part of the day, being returned periodically to the other flock and to the home flock, as shown in Tables 1, 2, and 3. The procedure was again repeated with certain hens, so that two hens became members in four flocks (Table 6) and four hens were rotated between five flocks (Table 7). During the second year the procedure was somewhat modified, so that the hens spent approximately equal amounts of time in all but the home flock (Tables 2 and 3).

Two hens from the same home flock were always moved into new groups together because it was thought that the introduction of two birds into a new group at the same time might facilitate their assimilation into the strange flock (cf. Allee, 1938). Throughout the first year of study the same two hens were partners in all flocks into which they were rotated as part-time members (Table 1). In the case of the death of one, the other hen of the pair was either moved alone or with another partner. During the second year of work all the hens of two different flocks, 6 and 8, were made absentee members of the home flock and were introduced into other hierarchies. The partners were changed so that the same two hens were not together in more than two flocks, although each was always with a member of its own home group (Tables 2 and 3). Only one pair of partners from a given home group was ever present at one time in a new group. The members of flock 6 were placed together and fed for 1 hour daily in pen 6 of the greenhouse. Those of flock 8 were fed together for 1 hour on alternate days in the front area of one of the henhouse pens.

A control group of nine hens was placed in the organized groups either individually, in pairs, or, in one case, in a group of three; and all became assimilated as permanently present resident hens. They were introduced by the same methods as experimental hens.

METHOD OF INTRODUCTION INTO A NEW FLOCK

At first, when new members were to be introduced, the original or resident members of a flock were moved from the pen or, in the case of those in the henhouse, were merely confined to the rear third of the pen by the inner doors, for about an hour. Beaverboard was placed over the screen of the doors, so that the residents could not see the strangers. During this hour the newcomers were placed in the pen and allowed to scratch around in it. Then the newcomers met all the resident hens at one time.

Gradually the technique was modified to determine whether, by spaced introductions to single members of the new

flock, the newcomers might attain a status in individual encounters which would later be maintained when they met the resident hens as a group. To observe this, it was arranged that the strangers meet each resident hen individually and on neutral territory in a series of spaced encounters so as not to tire the newcomers. A "neutral territory" is an area which is unfamiliar to all the hens concerned and was used because Schjelderup-Ebbe (1922) has shown that a member on home territory "wished" to fight in 93 per cent of the cases of first meetings, while the strange bird "wished" to fight in only 32 per cent of the cases, and that the home birds won 62 per cent of the observed battles. After the series of encounters which lasted from one day to one week, in an effort to prevent fatigue by repeated encounters, the newcomers were allowed to remain in the new pen for about an hour before meeting the resident hens as a group. The rotated flock members were always reintroduced after a routine period of absence by being placed on the roosts or nest ledges.

DAILY ROUTINE

During the first year the observations were taken in each flock with each set of part-time members for a period ranging from 10 minutes to 1 hour daily. It was soon noted that the greatest activity and the highest amount of pecking occurred during the first 10 minutes that the scratch food was present. Moreover, the rotated hens showed fewer reactions in the second flock if they had been fed for 1 hour in the first group, than they showed if they had been fed for only 10 minutes. It was, therefore, most satisfactory to observe any one flock for the first 10 minutes that the scratch food was present. After the first observation, in a pen with transient members, the food and the transients were removed. The part-time members were then placed in another flock with another hopper of food, and a second observation was taken. During the first year of study the visitors remained in the second pen for half an hour, during the second year for a full hour, before being removed either to a third flock or back to the flock where the first observation had been taken. During the first year, after half an hour in the third flock, the visitors were returned to the first group in which they had been observed that day (Table 1). Throughout the main part of the second year, the hens were allowed to remain overnight in the third flock as transients, so that the first observation the following day was taken where the last had been on the previous day (see Tables 2 and 3). This change was instituted for two reasons: (1) it equalized the amount of time spent in each flock visited, and (2) it allowed a more accurate study of the behavior of the transient members in each flock.

The observer watched the hens from a small stool placed in the doorway of the pens, later from outside the pens through the wire netting of the door. There was no indication that the hens were aware of the observer. The colored bands on the legs of the birds were clearly visible; and after a short period the observer came to recognize each hen without referring to the colors, although the identification bands were always checked for certainty. Details of behavior were recorded, as well as all pecks and threats. A threat was regarded as a peck when the hen at which it was aimed would have received the peck had it not dodged quickly.

Each day, the records of observations taken in the first flock, that is, where the transients had been present overnight, were kept separate from those taken in the second and third flocks into which

the hens were rotated. In the cases of the assimilated part-time members there was apparently no difference in the proportions of pecks given or received in the three instances.

ROTATION OF THE HENS

DURING THE FIRST YEAR OF STUDY

A total of ten hens was experimentally rotated from one flock to another during the first year of observations. Two of these became members of four flocks, one was a member of three groups, and the other seven were each moved between two flocks, as shown in Table 1. During the first year of study, flock i had four transients and two absentee members; flock ii had four absentees and two transients; flock iii, two absentees, two visitors, and four transients, two of which became visitors when the schedules changed; and flock iv, two absentees and four transients, two of which later became visitors.

DURING THE SECOND YEAR OF STUDY

In the henhouse.—During the second year the four hens comprising flock 8 were made absentees from the home flock and were introduced as part-time

TABLE 1

SCHEDULE OF ROTATIONS DURING THE FIRST YEAR OF STUDY

Hens*	Home Flock			New Flock into Which Introduced				Length of Time Spent in—			
	Group	Status	Total No. Hens	Date Introduced	Group	Status	Total No. Hens	Home Flock (Hours)	Second Flock (Hours)	Third Flock (Hours)	Fourth Flock (Hours)
RG	ii	2	6	Nov. 4	iii	2	9	½, daily	23½, daily	.....	.....
WW	ii	4†	6	Nov. 4	iii	3	9				
RG	ii	2	8	Mar. 30	iv	7	8	½, alt.§	½, daily	23, daily	.....
WW	ii	6‡	8	Mar. 30	iv	8	8				
RG	ii	6‖	10	May 10	i	1	6	½, alt.§	½, daily	½, alt.§	23, daily
WW	ii	7‖	10	May 10	i	2	6				
RW	iii	1	9	Oct. 12	i	4	5	½, daily	23½, daily	.....	.....
BB¶	iii	8	9	Oct. 12	i	5	5				
RW	iii	8‖	9	May 10	ii	10	10	½§	½§	23, daily	.....
LO	i	1	8	May 10	ii	9	10	½, daily	23, daily	.....	.....
PL**	i	1	8	Feb. 19	iv	2	7	½, daily	23½, daily	.....	.....
LO	i	2	8	Feb. 19	iv	3	7				
LW	ii	1	8	Apr. 4	iii	5	7	½, daily	23½, daily	.....	.....
BW	ii	5	8	Apr. 4	iii	6	7				
BO	iv	1	8	June 10	iii	9	10	23½, daily	½, daily	.....	.....
PR	iv	2	8	June 10	iii	10	10				

* Bracketed hens were moved together as partners.

† WW involved in a triangle, pecked by sixth hen.

‡ WW lost status to fifth hen, no longer in a triangle.

§ Indicates presence on alternate days only, other times indicate daily presence.

‖ Lost status since previous date.

¶ BB died March 14.

** PL died April 26, and after that date, LO, its partner to flock iv was no longer rotated into that group. Subsequently, LO was rotated into flock ii with a new partner, RW.

members into all four of the henhouse flocks. Throughout the following eleven-month period they met together for 1 hour on alternate days in the front area of a henhouse pen. Thus during the first period, as shown in Table 2, Y and PO spent the majority of the time in flock 1, while DW and B were in flock 3. During the second period Y and DW spent the greater part of each day in flock 4, while B and PO were in flock 2, and for 1 hour spent approximately one-third of its time as indicated in Table 2. In the fifth period DW and B were each members of five flocks, while Y and PO were rotated among only four. In the final period each hen was rotated among five flocks. (Y and DW were no longer partners in flock 4, nor were B and PO in flock 2.)

In the greenhouse.—Each member of flock 6 was rotated between flocks 5 and 7 and a small observation pen, with a different partner in each instance. Thus, during the first period, BY and RY were placed in flock 7, BR and BW in flock 5, and RL and LW in a small observation pen. During the second period the partners were changed as indicated in Table 3, and BY and RY were placed in flock 5, BR and LW in flock 7, and RY and BW in the small observation pen. They were present in those pens for 22 hours daily, were returned to the home flock and fed for 1 hour daily, and then were placed for 1 hour in the pens into which they

TABLE 2

SCHEDULE OF ROTATIONS OF HENS IN THE HENHOUSE DURING THE SECOND YEAR*

Hens	Flock into which introduced			Length of time spent in each flock according to periods					
	Flock	Status	Total No. Hens	Period 1 Oct. 24–Dec. 18	Period 2 Dec. 19–Jan. 6	Period 3 Jan. 7–Mar. 24	Period 4 Mar. 25–Apr. 22	Period 5 Apr. 23–May 2	Period 6 May 2–Sept. 8
Y and PO	1	5, 6	7	23 hours	1 hour†	1 hour†	⅓ time‡	¼ time	¼ time
DW and B	3	5, 6	7	23 hours	1 hour†	1 hour†	⅓ time	¼ time	¼ time
Y and DW	4	2, 3	9	.....	23 hours	alt. day	⅓ time	¼ time	.....
B and PO	2	1, 8	8	.....	23 hours	alt. day	⅓ time	¼ time	.....
Y and B	4	1, 2	7	.....	.....	alt. day	⅓ time	¼ time	¼ time
DW and PO	2	1, 8	8	.....	.....	alt. day	⅓ time	¼ time	¼ time
B and DW	1	4, 1	7	.....	.....	.....	.....	¼ time	¼ time
Y and PO	3	5, 7	7	.....	.....	.....	.....	¼ time	¼ time
Y and B	2	6, 7	8	.....	.....	.....	.....	.....	¼ time
DW and PO	4	1, 7	7	.....	.....	.....	.....	.....	¼ time

* All hens were absentee members of flock 8. For rank in home flock see Table 7.

† Alternate days only.

‡ The fractions indicate what part of the total amount of time was spent in a given flock. They are all approximate.

on alternate days each hen was rotated back to the flock into which it had been introduced during the first period. Throughout the third period the partners were again changed, so that Y and B were together in flock 4 while DW and PO were in flock 2 on alternate days, and Y and DW were in flock 4 while B and PO were in flock 2 on alternate days. Thus Y was in flock 4 and PO in flock 2 for the majority of the time, but with different partners every other day. During the fourth period each pair of hens

had been introduced the previous period. The rotations were changed during the third period, so that an equal amount of time, i.e., 23 hours on alternate days, was spent in both pens. In the final period each hen spent every third day in flock 5 with one partner, every third day with a second partner in flock 7, every third day in a small observation pen with a different partner, and 1 hour daily in group 6. At the end of this period the rotations were halted, and the hens remained in flock 6 permanently.

TABLE 3

SCHEDULE OF ROTATIONS OF HENS IN THE GREENHOUSE DURING THE SECOND YEAR*

Hens	Flock into which introduced			Length of time spent in each flock according to periods			
	Flock	Status	Total No. Hens	Period 1 Oct. 23–Dec. 21	Period 2 Dec. 22–Jan. 28	Period 3 Jan. 29–Apr. 10	Period 4 Apr. 11–May 8
BY & RY	7	5, 6	8	23 hours daily	1 hour daily	Alt. days	Every 3d day
BR & BW	5	4, 7	7	23 hours daily	1 hour daily	Alt. days	Every 3d day
RL & LW	Small observation pen			23 hours daily	1 hour daily	Alt. days	Every 3d day
BY & RL	5	6, 7	7	.....	22 hours daily	Alt. days	Every 3d day
BR & LW	7	7, 8	8	.....	22 hours daily	Alt. days	Every 3d day
RY & BW	Small observation pen			.....	22 hours daily	Alt. days	Every 3d day
RY & LW	5	5, 3†	6	.....	.....	.....	Every 3d day
RL & BW	7	7, 8	8	.....	.....	.....	Every 3d day
BY & BR	Small observation pen			.....	.....	.....	Every 3d day

* All hens were absentee members of flock 6. For rank in home flock see Table 9.
† LW achieved gamma rank during initial encounters only (see Table 9).

RESULTS

BEHAVIOR AT FIRST ENCOUNTERS

During the first encounters with resident hens as a group or individually, the newcomers either fought, passively submitted to being pecked, or avoided the residents, as has been described by Schjelderup-Ebbe (1935). Thus, from Table 4, out of thirty-six introductions in which newcomers met the residents as a group, the strange hens in twenty-six cases fought one or more of the residents, four hens avoided all contact during the observation, four more submitted to being pecked, and two cases were not observed. Thirteen newcomers met the resident hens in individual encounters, while four were placed with strange hens which were being kept together for the first time as a new flock.

When a fight occurred between newcomers and resident hens, the dominant resident hen was invariably involved. Thus in 14 per cent of the cases the dominant was the first hen to challenge one or both of the strangers; in 36 per cent the alpha resident interfered in fights between newcomers and more subordinate residents; and in 36 per cent the alpha was the second resident to challenge the strange birds. In the remaining 14 per cent of the cases one of the strangers attacked the alpha resident hen first. Usually one partner of the newcomers stood near by while the other fought. If the fighting stranger won the fight, its partner usually attacked the losing resident and frequently became dominant to it also. If the fighting partner lost, the other of the newly introduced pair might passively submit to the victor, although in four cases one partner

fought and the other simultaneously submitted to being pecked or avoided all contact with the resident hens during the observation. The first pair-contacts were apparently conclusive, for, in the cases observed, the newcomers retained the status achieved during initial encounters. This has occurred in too few cases to enable one to draw any general conclusion without reference to the supporting literature (cf. Schjelderup-Ebbe, 1922, 1935).

TABLE 4

METHOD OF INTRODUCTION OF STRANGERS, INITIAL REACTIONS, AND STATUS ACHIEVED*

Reactions of Newcomers	Type of Introduction						Total
	Newcomers Met Residents as a Group				Individual Encounters	With New Flock†	
	Fight	Avoid Contact	Submit to Pecks	Not Observed			
Number dominant	7	.....	.....	2	3	.....	12
Number subordinate	16	3	4	.....	6	2	31
Number not assimilated	3	1	.....	.....	4	2	10
Total	26	4	4	2	13	4	53

* This includes the introductions of the nine hens which became residents in one flock only.

† The expression "with new flock" is used to indicate that the group was not organized at the time that the part-time members were placed in it.

FACTORS AFFECTING TIME OF ASSIMILATION

The process of assimilation involves an interaction between stranger and resident hens. The latter gradually allow the newcomers more freedom of movement, feeding, nesting, and other flock activities than they accord to unassimilated individuals. At the same time, the newcomers, on their part, show some degree of aggressiveness in joining in flock activities in spite of the pecks of the resident members, for a hen which continuously avoids all resident hens does not become assimilated. Thus the residents gradually become more tolerant of the newcomers, and, simultaneously, the hens in the process of assimilation tend to join with the group more freely.

A subordinate newcomer which is becoming assimilated gradually begins to feed and mingle with the others; and, finally, after a period varying from 3 to 6 weeks, there is no apparent difference between old and new members of the group with similar social rank, individual differences aside. On the other hand, if the newly introduced individuals become relatively dominant in the social hierarchy, then they can be considered assimilated as soon as they allow the subordinates to feed as freely as do most dominant residents (Table 5). Again there is some individual variation among the hens, but, on the whole, the assimilation time in these cases is within 3 days of the first introduction. Thus the status achieved after being placed in the group is an important factor in the length of time required for assimilation into flock membership.

We shall now examine other factors to determine their effects on (1) the status of the newcomer after introduction and (2) the time required for assimilation.

The method by which a hen was introduced to the resident members apparently had no effect on the status achieved after introduction and also did not affect the length of assimilation time. Table 4 shows that seven hens, or 27 per cent of the newcomers which met the resident hens as a group, became relatively dominant, while three hens, or 23 per cent of those which met residents in individual pair-encounters, became relatively dominant, that is, in the alpha, beta, or gamma position. Of the strangers which met the resident hens as a group, 62 per cent became relatively subordinate and 12 per cent not assimilated, while, of the small number which met in individual encounters, 46 per cent became subordinate, and 30 per cent were not assimilated. It is unlikely that the method of introduction caused the higher proportion of non-assimilated individuals among those which met each member of the group individually, since those which did not become assimilated all belonged to flock 6, in which lack of assimilation may be associated with other factors discussed elsewhere.

Table 5 shows the approximate time of assimilation, method of introduction, ini-

TABLE 5

METHOD OF INTRODUCTION, STATUS AFTER INTRODUCTION, AND APPROXIMATE TIME OF ASSIMILATION

Hen	New Group into Which Introduced				No. Flocks in Which Already a Member
	Flock	Method	Relative Initial Status*	Approximate Time of Assimilation	
New Resident Members Introduced into One Flock Only					
PW	i	Group†	Sub.	4 weeks	None
PG	i	Group	Sub.	4 weeks	None
GG	i	Group	Sub.	4 weeks	None
LL	ii	Group	Sub.	5 weeks	None
LY	ii	Group	Sub.	5 weeks	None
PL	iv	Ind.‡	Dom.	Within 3 days	None
TG	3	Ind.	Sub.	4 weeks	None
DY	3	Ind.	Sub.	4 weeks	None
BYR	4	Ind.	Dom.	Within 3 days	None
Hens Experimentally Rotated during the First Year§					
RG	iii	Group	Dom.	Within 3 days	One
WW	iii	Group	Dom.	Within 3 days	One
RG	iv	Group	Sub.	5 weeks	Two
WW	iv	Group	Sub.	5 weeks	Two
RG	v	Group	Dom.	Within 3 days	Three
WW	v	Group	Dom.	Within 3 days	Three
RW	i	Group	Sub.	5 weeks	One
BB	i	Group	Sub.	5 weeks	One
RW	ii	Group	Sub.	5½ weeks	Two
LO	ii	Group	Sub.	5½ weeks	One
PL	iv	New flock	Dom.	?	One
LO	iv	New flock	Dom.	?	One
LW	iii	Group	Sub.	4 weeks	One
BW	iii	Group	Sub.	4 weeks	One
BO	iii	Group	Sub.	?	One
PR	iii	Group	Sub.	?	One

Hen	New Group into Which Introduced				No. Flocks in Which Already a Member
	Flock	Method	Relative Initial Status*	Approximate Time of Assimilation	
Members of Flock 8 Experimentally Rotated‖					
Y	1	Group	Sub.	4 weeks	One
PO	1	Group	Sub.	4 weeks	One
B	3	Group	Sub.	3 weeks	One
DW	3	Group	Sub.	3 weeks	One
Y	4	Group	Dom.	Within 3 days	Two
DW	4	Group	Dom.	Within 3 days	Two
B	2	Group	Dom.	Within 3 days	Two
PO	2	Group	Sub.	6 weeks	Two
B	4	Group	Dom.	Within 3 days	Three
DW	2	Group	Dom.	Within 3 days	Three
Y	3	Ind.	Sub.	6 weeks	Three
PO	3	Ind.	Sub.	6 weeks	Three
B	1	Ind.	Sub.	1 week	Four
DW	1	Ind.	Dom.	3 days	Four
PO	4	Group	Sub.	5 weeks	Four
Y	2	Ind.	Sub.	4 weeks	Four
Members of Flock 6 Experimentally Rotated¶					
BR	5	Group	Sub.	4½ weeks	One
BW	5	Group	Sub.	4½ weeks	One
BY	7	New flock	Sub.	With flock	One
RY	7	New flock	Sub.	With flock	One
BY	5	Group	Sub.	4½ weeks	Two
RL	5	Group	Sub.	4½ weeks	One
BR	7	Group	Sub.	?	Two
LW	7	Group	Sub.	?	One
RY	5	Ind.	Sub.	?	Two
LW	5	Ind.	Dom.	?	Two
RL	7	Ind.	Sub.	?	Two
BW	7	Ind.	Sub.	?	Two

* Alpha, beta, and gamma positions were considered relatively dominant and are indicated as "Dom."; other positions were relatively subordinate and are indicated as "Sub."

† The term "Group" indicates that the newcomers met all the resident hens at one time.

‡ The term "Ind." indicates that the newcomers met each resident hen in individual pair-contacts before meeting them as a group.

§ For details see Table 1. ‖ For details see Table 2. ¶ For details see Table 3.

tial status, and number of flocks in which the hen was already a member, for all the hens rotated during the two years studied. The time required for assimilation varied from within 3 days of introduction for relatively dominant hens to from 3 to 5 weeks for those relatively subordinate. It is therefore obvious that the status achieved in the social hierarchy is more important in determining the assimilation time than is the method of introduction.

TABLE 6

SOCIAL STATUS OF **RG** AND **WW** WHEN MEMBERS OF FOUR FLOCKS SIMULTANEOUSLY

Rank in Social Hierarchy	Flock			
	i	ii	iii	iv
	May 10–July 12	Oct. 6–July 12	Nov. 4–July 12	Mar. 30–July 12
1	**RG**	LW	BY	**RG**†
2	**WW**	**RG**	**RG**	BO
3	PY	GY	**WW**	PL‡
4	PW	**WW***	RR	PR
5	PG	BG	BR	YY
6	GG	BW*	RY	GL
7	.....	LY	RL	YW‡
8	.....	LL	RW	**WW**
9	.....	.....	BO	.....
10	.....	.....	PR	.....

* BW pecked **WW**.

† **RG** was subordinate in flock iv when first introduced but later became dominant.

‡ YW pecked PL.

The number of flocks in which a hen was already a member had no effect on the length of time required for assimilation into a new group, as can be seen in Table 5. A comparison between the new resident hens which never had been placed in more than one group and those already members of more flocks shows no apparent difference in the assimilation time. Moreover, the status achieved by a hen in a new flock was also apparently unaffected by the mere number of flocks in which it had previously been a member.

The conditioning which occurred in connection with social status in previous flocks might have affected the status in the new flock and thus indirectly the time required for assimilation (cf. Collias, 1943). Hens higher in the hierarchy of previous groups tended to fight harder and longer than did those in subordinate positions. For example, PO, omega in flock 8 (Table 7), avoided contacts whether it met the strange residents as a group or individually. On the other hand, RG, beta in the home flock, ii (Table 6), and Y, alpha in the home flock (8) (Table 7), both fought while being introduced into new groups. Table 4 summarizes the relations between reactions at first encounters and subsequent status in the group.

In spite of the possible effects of conditioning on the achievement of status in the new group, it was apparently impossible to predict the rank of a newcomer in a flock from a knowledge of its status in other flocks. Thus rotated hens usually did not hold the same social position in each flock in which they were members. The social status of RG and WW while simultaneously members of four flocks is shown in Table 6. In the home flock, ii, they were second and fourth, although WW was involved in a triangle in which it was pecked by the sixth hen. In the second flock, iii, they were second and third for a period of 9 months. In the third group, iv, they were at first seventh and eighth, later alpha and eighth, for a total of $3\frac{1}{2}$ months. They were alpha and beta in the fourth group, i, for 2 months and also throughout the entire second year of study. Thus RG was alpha or beta in each of the four flocks (except for a short while when it was seventh in flock iv), while WW held ranks ranging from beta to omega.

Similar variations in status among the members of flock 8 are shown in Table 7.

The alpha hen of flock 8, Y, was fifth in flock 1 for 11 months, sixth in flock 2 for 4 months, fifth in flock 3 for 4½ months, and beta—then alpha (when the resident alpha died)—in flock 4. Hen B, beta in flock 8, was fourth in flock 1, alpha and then seventh in flock 2, seventh in flock 3, and beta in flock 4. DW and PO held the indicated social positions shown (except that DW lost status in three flocks, possibly because of a respiratory infection discussed elsewhere) until the final month of rotations, when each received a pellet of testosterone propionate implanted subcutaneously and rose in social rank. From Table 5 it can be seen that the length of time required for the assimilation varied with each hen in different flocks. Thus in flock 3, where DW and B were both subordinate, the assimilation time was approximately 3 weeks, while in flock 4, although present with different partners, both were relatively dominant and assimilated within 3 days of introduction.

Thus the status of newcomers was ap-

TABLE 7

STATUS OF MEMBERS OF FLOCK 8 WHILE ASSIMILATED MEMBERS OF FIVE FLOCKS
(OCTOBER 9, 1944—SEPTEMBER 8, 1945)
(Social Rank in Flock 8: Y > B > DW > PO)

Rank in Social Hierarchy	Flock 1			Flock 2			
	Part-Time Members			Part-Time Members			
	None	Y & PO	DW & B	None	B & PO	B & Y	DW & PO
1	GY	GY	**DW***	BYT	**B**	BYT	**DW***
2	BG	BG	GY	L	BYT	L	BYT
3	RW	RW	BG	BP	L	BP	L
4	LY	LY	**B**	RR	BP	RR	BP
5	LO†	**Y**	RW	RYT	RR	RYT	RR
6	.....	**PO**	LY	W	RYT	**Y**	RYT
7	.....	LO†	LO†	PW‡	W	**B**§	W
8	.....	.....	.....	.....	**PO**	W	**PO**

Rank in Social Hierarchy	Flock 3			Flock 4			
	Part-Time Members			Part-Time Members			
	None	DW & B	Y & PO	None	Y & DW	DW & PO	Y & B
1	BW	BW	BW	LL‖	LL‖	**DW**	**Y**
2	GW	GW	GW	TW	**Y**	TW	**B**
3	TG	**DW***	TG	YY	**DW**	YY	TW
4	DY	TG	DY	DR	TW	DR	YY
5	YW	DY	**Y**	BB‖	YY	YO	DR
6	.....	YW	YW	YO	DR	O	YO
7	.....	**B**	**PO**	O	BB‖	**PO**	O
8	.....	.....	.....	.....	YO	.....	.....
9	.....	.....	.....	.....	O	.....	.....

* **DW** lost status in the three flocks indicated.
† LO pecked RW.
‡ PW died February, 1945.
§ **B** pecked BYT.
‖ Birds died: LL, May 17; BB, June 6.

parently unaffected by the method of introduction or by the number of flocks in which each was already a member but was influenced by previous conditioning and by individual differences between hens, both newcomers and residents. The length of time required for assimilation into a new group depended on the status achieved after introduction more than on any of the other factors analyzed.

INTERACTIONS AFTER ASSIMILATION

Behavior.—The resident hens permanently present reacted toward assimilated part-time members in the same fashion as toward resident hens holding approximately equal social status. Similarly, there was no observed difference in the behavior of the part-time hens when compared with behavior of resident hens at about the same rank in the hierarchy. When first returned after a period of absence, by being placed on the roosts, relatively dominant hens immediately flew to the floor and wandered about the pen, pecking subordinate birds and feeding, while relatively subordinate individuals tended to linger on the roosts for a short while and sometimes were chased to the floor by more dominant residents. On the floor they reacted subordinately to the more dominant birds and pecked any to which they were dominant.

Pecking proportions.—It is perhaps significant that these observed similarities in behavior between resident and part-time members with similar rank are apparently reflected in the proportions of the total pecks given and received by hens of a similar status in any one flock. Some pecking proportions computed from records of the second year of work are shown in Table 8. The data cover observations taken after the assimilated transients had been present with the resident members of the flock overnight. In the first analysis the year was divided into periods according to the number of flocks in which each experimental hen was present, as shown in Table 2. No statistically significant difference could be found between the periods, perhaps because of the wide variability from observation to observation. Most fluctuations could not be correlated with any apparent changes in the behavior of the hens, apart from the pecking. Consequently, it was thought best to combine all observations made on a given pair of transients in any flock over the entire period of time that they were members of the flock.[4] The importance of the figures used lies in their correlation with the observed behavior of the individuals. More than an auxiliary reliance on these figures would seem unwise under any circumstances, since the proportions of pecks delivered and received depends as much on avoidance tendencies of subordinate birds as it does on aggressive behavior by dominant ones.

The results in Table 8 are arranged according to flocks, and the hierarchies indicated are the same as those shown in Table 7. There is apparent some tendency toward a constant percentage of pecks delivered and received in a given rank in any one flock. Individual differences in aggression and avoidance make it more difficult to compare one flock with another.

A study of the proportion of pecks delivered by relatively dominant hens shows few differences between residents and transients. In flock 1 the dominant hen delivered 27 per cent of the total number of pecks, whether the residents were without part-time members or whether relatively subordinate transients

[4] The figures for the final month, after DW and PO each had received a pellet of testosterone propionate, are omitted in the flocks in which they are present.

TABLE 8

PECKS DELIVERED AND RECEIVED BY HENS ACCORDING TO RANK

(Figures are in percentages of the total pecks for a given flock. Boldface figures indicate those of part-time members)

FLOCK	PART-TIME MEMBERS	DURATION OF OBSERVATIONS (MONTHS)	FLOCK HIERARCHIES*																	
			Dominant		Second		Third		Fourth		Fifth		Sixth		Seventh		Eighth		Ninth	
			Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.
1†	None	3	27	.....	13	1	8	42	11	2	40	56	.....	.....	.....	.....	.....	.....	.....	.....
	Y & PO	10	27	.....	17	1	12	25	10	3	**10**	**19**	**3**	**19**	21	33	.....	.....	.....	.....
	DW & B	4	**24**	**19‡**	10	6‡	10	7	**23‡**	**17**	2	17	25	4	5	30	.....	.....	.....	.....
2	None	3	29	.....	24	3	18	11	16	11	9	26	5	27	.....	22	.....	.....	.....	.....
	B & PO	5	**55**	.....	8	10	16	7	3	10	13	13	2	27	2	9	.....	**24**	.....	.....
	Y & B	5	22	**2§**	13	2	5	5	20	7	27	13	**7**	**37**	**6§**	**18**	.....	17	.....	.....
	DW & PO	4‖	**52**	.....	21	14	7	8	5	11	8	14	6	17	1	10	.....	**27**	.....	.....
3	None	3	34	.....	15	1	38	13	12	12	.....	72	.....	.....	.....	.....	.....	.....	.....	.....
	DW & B	10	18	.....	18	1	**17**	**31**	12	8	20	2	10	32	**6¶**	**27**	.....	.....	.....	.....
	Y & PO	4	26	.....	15	1	18	4	11	8	**17**	**28**	12	35	.....	**24**	.....	.....	.....	.....
4	None	3	36	.....	14	10	22	14	16	14	5	11	7	23	.....	29	.....	.....	.....	.....
	Y & DW	5	20	.....	**18**	**2**	**24**	**7**	6	15	10	14	16	18	3	5	2	12	1**	26
	DW & PO	5	**34**	.....	16	5	23	10	19	17	4	20	5**	34	.....	**14**	.....	.....	.....	.....
	Y & B	5	**22**	.....	**45**	**3**	16	12	9	18	6	18	2	22	1**	28	.....	.....	.....	.....

* Flock hierarchies refer to Table 7. All figures are from observations taken after part-time members had been present for approximately 22 hours previously.

† Flock 1 had a triangle in which the fifth hen pecked the third.

‡ Transient hen B, fourth in the hierarchy, pecked the second hen; and DW, alpha transient, lost status and was pecked by its partner and two resident hens.

§ Transient hen B pecked the alpha resident.

‖ Observations after DW lost status not included.

¶ Transient hen B, seventh, pecked its partner, third in the hierarchy.

** Omega resident hen delivered approximately 1 per cent of the pecks to its superiors.

were present. Similarly, the alpha transient delivered 24 per cent of the total, although involved in a triangle. In flock 4 without transients the dominant delivered 36 per cent of the total, while one alpha transient delivered 34 per cent and another 22. The pecking of the alpha hen of flocks 2 and 3 was of approximately the same order of magnitude, although the transient dominants delivered a somewhat higher proportion in flock 2. This variation may be no greater than the individuality of the hens might allow, or it may be some evidence of increased tension, especially since both these dominant transients later lost status in that flock—B when its partner in the group was changed from PO to Y, and DW probably as a result of the respiratory infection which made breathing difficult.

The assimilated subordinate part-time members also delivered a proportion of the pecks which was not essentially different from that delivered by resident hens with similar status. For example, in flock 1, when Y and PO were present, Y, two ranks above the omega in the hierarchy, delivered 10 per cent of the total, while the third from the omega resident in the same social position as Y delivered 8 per cent when no part-time members were present. This case is, however, complicated by the fact that the fifth resident pecked the third in a triangle relationship. In flock 2, Y was two ranks above the omega and delivered 7 per cent, while the resident, with similar social status, gave 9 per cent. When DW and B were present in flock 3, the fifth place in the hierarchy was occupied by a resident hen, which delivered 20 per cent of the total pecks, while, when Y and PO were the transients, Y was in the fifth place and delivered 17 per cent of the total. Comparisons between the beta and gamma hens in flock 4 show similarities between the proportions given and received by transients and residents, although in one case the transient delivered more than twice the proportion of the others. This hen, B, also delivered a higher proportion in flock 2 when it was alpha transient there. Close scrutiny of the table will show some cases which apparently do not conform with the above discussion. As stated above, this may be the result of individual variations. Of the thirty-two such comparisons between proportions of pecks delivered by residents and part-time members, in four cases the transient hen delivered a relatively high proportion for its rank in the group. Three of these cases involve hen B; the fourth concerns hen DW, although B and DW did not always react so. These exceptions may well indicate increased tensions with rotating membership.

In a similar fashion, of thirty-two comparisons of proportions of pecks received by the two classes of hens (triangles aside), in only two cases (hen Y in flocks 2 and 3) did the transient receive a somewhat higher proportion of the pecks than did a resident with similar status. For example, in flock 2, with PO present and omega, it received 27 per cent of the total when its partner was DW, and 24 per cent when B was present, while the resident omega hen received 22 per cent of the total, when no part-time members were in the group. In the same flock, when Y and B were present and sixth and seventh, respectively, they together received 55 per cent of the total, while the fifth and sixth resident hens (i.e., second and third from the omega, as were the transients) together received 53 per cent when no transients were present.

Further instances can be seen in flock 4, where the transients were relatively dominant. In this case it is evident that they were not receiving discriminatory pecking from the alpha resident hen. In

the same flock PO received approximately half the amount received by the resident omega. This is because, whenever a resident pecked PO, its dominant partner, DW, would peck that resident. This caused the resident members to avoid pecking PO. In flock 3 the resident omega received 72 per cent of the total pecks when no transients were present, but less than half that amount when omega transients were in the group. Thus, apparently, the pecking of the more dominant individuals was divided between the resident omega and the transient omega hens when the latter were present.

These results have shown that the proportion of pecks received by transient members was approximately the same as that received by resident hens with similar status. Similarly, the proportion of pecks delivered by transient members usually approximates the proportions given by resident hens with comparable status. This, correlated with the observed behavior, seems to confirm the statement that the assimilated part-time individuals were treated as any resident member of the social group with similar hierarchy status.

Maintenance of status.—Assimilated hens tended to maintain for long periods of time the same status as that achieved after initial introductions, although there were some exceptions. Of the forty-four introductions of hens which were already members of one or more flocks, thirty-four resulted in hens becoming assimilated into the new group. In twenty-one of these cases there was no change in status throughout the period of rotation, a total of from 1 to 10½ months. In five cases part-time members became more dominant after assimilation; two over a resident hen which became ill and died shortly afterward; one was associated with a change in the rotation schedule; and one case (RG, Table 6, flock vi) became dominant to all resident hens after becoming dominant to the alpha and beta residents when the latter were rotated into another group. In five cases the part-time members lost status achieved during initial encounters. Three of these involved the hen that had developed a severe respiratory infection which made it difficult for the bird to breathe. This hen lost to one resident in one flock, to two in a second group, and to four in a third flock (DW, Table 7). The other two losses of status were probably associated with a change in the schedule of rotations. In three cases the apparently assimilated individuals lost status and simultaneously lost assimilation in the flock. These will be discussed below.

Each of the twenty experimental hens introduced into new flocks were also absentees from a home flock. Fifteen of these maintained their rank in the home flock for periods of from 1 to 11 months during which observations continued. Each of the other five lost status shortly after a change in its rotation schedule. Table 1 indicates the change in rank for RG and WW in flock ii and for RW in flock iii. A fourth hen, LO, beta in its home flock, i, maintained status there while it was an absentee member with PL as its partner but later lost status when RW became its partner. The fifth hen to lose status in its home flock was BR in flock 6 (Table 9).

It would therefore appear that continuous presence with its flockmates is not essential for maintenance of flock membership and status in the flock hierarchy, provided that the part-time member is present at intervals. In no case was a part-time member absent from a flock for more than 48 hours (that is, it was returned at least every third day), while in most cases the interval was shorter. The length of the intervals of absence which would allow retention of status

was not determined, nor was the length of needed presence at any given time. Dr. A. M. Guhl (personal communication) stated that the hierarchy in his flock of ninety-seven hens was not so stable as that in smaller flocks. The fact that there was more change of status among experimentally rotated hens than there was among the permanent residents may indicate a similarity between being rotated among a fairly large number of individuals and being in a large group continuously. Those in a flock of almost one hundred individuals might not always be in the presence of all other birds, and thus two days or more might pass when two individuals were not in actual contact. This might be a factor in overcoming the social inertia which makes small flock hierarchies stable over long periods of time, even when one or more members are not healthy.

The introduction or the removal of part-time members had no effects on the hierarchical relations of the resident hens in the ten flocks with permanently present members, except in one case. At that time an alpha resident hen became subordinate to two newly introduced transient hens, and within 48 hours of their introduction it had become omega in the whole hierarchy. In all other cases, regardless of the social status of the newcomers, the relations of the residents toward one another were unchanged. Thus the alterations in the size of the flock with the addition or removal of part-time members and the change in the membership of the flocks apparently did not affect the basic hierarchical organization.

APPARENTLY UNASSIMILATED HENS

From Table 5 it can be seen that some of the rotated hens when introduced into new flocks apparently did not become assimilated. These hens were frequently chased by the resident hens from one area of the pen to another. They were not allowed to feed with the other members, and they tended, furthermore, to remain in one corner and to avoid all contacts. Thus they did not mingle or attempt to mingle with the residents as did newcomers which were becoming assimilated. Moreover, the resident hens pecked and chased these individuals more than they did resident hens with similar status. In some cases the apparently unassimilated individuals received all the pecks delivered throughout the period of observations.

During the first year of study one pair of experimental hens, PL and LO, failed to become assimilated in flock iv (Table 5). These individuals were introduced from an organized henhouse flock into a group of hens in the greenhouse, which had just been received in the laboratory and had not yet formed an organized flock with a distinct hierarchy. It is possible that the different conditions of housing in the two cases might have resulted in a lack of adjustment. Within 6 weeks of the introduction, one of these unassimilated newcomers sickened and died. The other member of the pair, LO, had gone into a moult, and, when the resident members of the flock started to peck at its bare back, it apparently lost status. Thus physiological changes in the two hens might account for their lack of assimilation.

The other two hens which had not become assimilated by the close of the experiments during the first year of study were BO and PR, visitors to flock iii for half an hour on alternate days for a period of approximately one month. Since this is not more than the time required for assimilation of hens which are present for a large part of the time, it is not known whether they would have become assimilated if the rotations had been continued.

During the second year of study five of the six absentee members of flock 6 failed to achieve assimilation in one or both of the two new flocks into which they were introduced. Furthermore, these hens appeared to lose assimilation in the flocks in which they had formerly been judged to be accepted members. The causes of failure to become assimilated, or of its apparent loss when attained, are difficult to ascertain. An attempt to determine the reasons by a comparison between conditions affecting flock 6, whose members lost or did not achieve assimilation, and conditions affecting flock 8, whose members achieved assimilation, follows. It will be recalled that all the members of both flocks were absentees, spending almost all of their time away from the home flock.

1. Instead of being moved from one flock to another so that they were continuously with an organized group, the hens of flock 6 were each rotated with a partner into one of the small observation pens described above (cf. p. 150). This confinement appears in most instances to be associated with subsequent failure to become assimilated. The first pair of hens to be so confined was RL and LW (Table 3), and LW[5] failed subsequently to become assimilated into either of the new flocks, 5 or 7, into which it was introduced. Hen RL, on the other hand, became apparently assimilated into flock 5, where its partner was BY, but did not become assimilated into flock 7. The second pair of hens rotated into the observation pen was RY and BW. Both of these had become assimilated in the strange flock into which each had been introduced during the previous period, and both failed of assimilation in the new

TABLE 9

SOCIAL STATUS OF HENS OF FLOCK 6 WHEN MEMBERS OF THREE FLOCKS SIMULTANEOUSLY (OCTOBER 9, 1944—MAY, 1945)

(Social Rank: BY* > BR† > RY‡ > RL > LW§ > BW)

Rank in Social Hierarchy	Flock 5				Flock 7			
	Part-Time Members				Part-Time Members			
	None	BR & BW	BY & RL	RY & LW	None	BY & RY	BR & LW	RL & BW
1	RG	RG	RG	RG	PY	PY	PY	PY
2	WW	WW	WW	WW	GO	GO	GO	GO
3	PY‖	PY‖	PY‖	PG	PR	PR	PR	PR
4	PG	**BR**¶	PG	GG	GY	GY	GY	GY
5	GG	PG	GG	**RY**	BO	**BY**††	BO	BO
6	.....	GG	**BY**	.....	GL	**RY**††	GL	GL
7	.....	**BW**	**RL**	**LW?****	.....	BO	**BR**	**RL**
8	.....	.....	.....	.....	.....	GL	**LW**	**BW**

* Died July 24.

† Lost status and later died on May 11.

‡ Died July 21. § Died June 18. ‖ Died March 23.

¶ **BR** lost status during the last period.

** **LW** became third in individual encounters but was never observed to peck during its month in flock 5; therefore its true status is unknown.

†† Later lost status.

[5] The previous year this same hen had been rotated from flock ii to flock iii, where it had apparently become assimilated while still maintaining its alpha status in the home flock (Table 1). Flock iii became flock 6 during the second year, and LW was apparently still assimilated in the group.

flock into which they were introduced after their rotation into the small pen. The last pair placed in the small pen was BY and BR. The former had already, before this period, become assimilated into both flocks 5 and 7, but BR only in flock 5. Now each bird of flock 6 was spending every third day in the observation pen, and every third day in flock 5 or 7. It was during this last period that even those individuals which had apparently achieved assimilation began to react in the typical fashion described for unassimilated hens. These avoidance reactions were so marked that those hens which had achieved assimilated status above the rank of omega lost that status, as indicated in Table 9. In addition, the reactions of the resident hens toward the transients also changed so that the latter were frequently chased around the pens in a manner apparently identical to that shown toward unassimilated newcomers.

These results seem to be similar to those of Guhl and Allee (1944), who reported that hens which were moved from one flock to another adjusted to the new groups more easily than did those which were moved from the isolation pens to the groups. Thus the time spent away from the group might have decreased the ability of the individuals to adjust to other hens when returned to the group.

2. The loss of assimilation of the hens of flock 6, which occurred, we have seen, when each member was spending one day out of every three in each flock it was visiting, might indicate that the hens failed to remember each other after an absence of 48 hours. Either the rotated members of flock 6 or the residents of flocks 5 and 7 might have been unable to recognize other hens which had been out of sight for 2 days. It is interesting to note, however, that the members of flock 8, which were rotated into four flocks, were able to maintain assimilation under a similar time schedule (Table 2, period 4).

3. Conditioning to feeding in the home flock or in the small observation pen instead of with the resident members of flocks 5 and 7 may also be a factor in the loss of assimilation of the rotated hens. Such conditioning may account for the failure of the hens of flock 6 to attempt to feed with resident hens, a failure which is in itself one of the criteria for nonassimilation. Thus the members of flock 6 were fed for 10 minutes in the pen in which they had spent the previous day (5, 7, or the observation pen) and then for a full hour in the home flock, before being rotated again. In contrast to this, the members of flock 8 met together for only 1 hour on alternate days and were otherwise always fed in one of the other flocks. It is possible that these hens did not become conditioned to feeding in the home group and therefore tended to mingle with and feed with the resident hens of the other groups into which they were placed.

4. At the start of the experiments all the hens were apparently in good health. The members of flock 8 (with the exception of DW, which developed a severe respiratory infection and lost status in three flocks probably as a consequence), remained healthy throughout the course of the rotations. On the other hand, four of the members of flock 6 appeared ill, and all four died within two months of the close of the experiment. It is possible that certain physiological changes preceded these appearances of ill health among the birds and that these affected the social relations between the hens; such loss in status does not always obtain when birds die (Allee, Collias, and Lutherman, 1939). It is known that physiological changes which accompany moulting can affect the outcome of individual pair-contacts (Collias, 1943), and there-

fore it is possible that physiological changes, not apparent at first to the observer, preceded the changes in behavior.

5. There was a difference in the housing of the hens of flock 8 which became assimilated in the four new flocks, all in the henhouse, and the housing of the members of flock 6. The henhouse pens were all similar and undisturbed except when the observer or the cleanup man was present. In the greenhouse the pens were not alike, and all bordered on a path which many people used in moving about the greenhouse. Thus there were constant disturbances from noise and unfamiliar individuals moving past the walls of the pens. The lighting conditions were controlled in the henhouse by means of a time clock, while this was impossible in the greenhouse.

6. The matter of individual differences in ability to adjust to the rotations and to the changed conditions must not be overlooked. It is possible that the members of flock 6 which were unable to adjust became ill and that this, in turn, might have affected their ability to remain assimilated in the various flocks.

7. The final period during which the members of flock 6 were rotated was approximately 4 weeks in length. It has been shown that relatively subordinate newcomers require from 3 to 6 weeks after introduction before they can be considered assimilated members of the new group. It is therefore possible that certain of the hens might have become assimilated, had the experimental rotations been continued for a longer period of time. This seems improbable for BR, which died immediately after the close of the rotations, and even for LW, which died 5 weeks later.

Thus the failure to become or remain assimilated may be caused by rotation into a small confined observation pen, away from contact with an organized flock; the length of absence from the group; conditioning to being fed in the small observation pen or the home flock; physiological changes attendant on ill-health; differences in housing conditions; or individual differences in ability to adjust to changing conditions. It is obvious from the cases at hand that it is impossible to draw any conclusions concerning which factor is the most important in the inability of certain individuals to achieve or maintain assimilated status. It is highly possible that there is an interrelation between the factors, some or all of which may be partly responsible.

Pecking proportions.—It has been shown that resident hens behave more aggressively toward nonassimilated than toward assimilated members of the group. Unassimilated hens react toward the residents with strong avoidance tendencies. Apparently correlated with this observed behavior are the pecking proportions. Assimilated part-time members of the group, as indicated above, neither deliver nor receive a higher proportion of the total pecks than do resident members with similar status. This is not true of unassimilated individuals, as is strikingly shown in flock 5 by comparing the periods when the part-time members were apparently assimilated with those when they were apparently not so. Thus, for example, during the first 6 months that BR and BW were present in flock 5 and apparently assimilated, they received together only 45 per cent of the total pecks delivered. This is slightly less than the omega resident hen received when no transients were present. However, during the last month in the flock, when they were judged to have lost assimilation, together they received all the pecks delivered throughout each observation. Similarly, when BY and RL were considered assimilated during the first 4 months of

their rotations into flock 5, they received 42 per cent of the total pecks, while during the last month, when they were considered unassimilated, they received 83 per cent of the total. When RY and LW were first introduced after a period of absence they also received all the pecks delivered.

In flock 7, on the other hand, only one pair of the part-time unassimilated hens, RL and BW, together received 100 per cent of the pecks when first returned after a period of absence. The lower pecking figures for the other hens undoubtedly reflect the strong and successful avoidance reactions observed on the part of the nonassimilated part-time individuals.

TABLE 10

PECKS DELIVERED AND RECEIVED ACCORDING TO RANK IN FLOCK 8

(Figures are in percentages of the total pecks for a given period)

Dates*	Y		B		DW		PO	
	Rank							
	1		2		3		4	
	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.
First 2 weeks	38	.....	42	22	21	58	.....	33
Oct. 24–Dec. 18	88	.....	2	71	4	16	6†	13
Dec. 19–Jan. 6	90	.....	.....	19	10	10	.....	71
Jan. 7–Mar. 24	76	.....	16	24	9	16	.....	60
Mar. 25–Apr. 22	73	.....	5	50	23	17	.....	33
Apr. 23–Aug. 11‡	73	.....	11	31	16	25	.....	44
Oct. 24–Aug. 11	79	.....	9	44	12	21	2†	35

* Dates refer to periods shown in Table 2.

† On one day, Y pecked B severely, and B submitted in one corner of the pen. Before it left that corner, PO pecked it several times. It was not observed to peck B again, however.

‡ Behavior changes induced after this date by the implantation of the testosterone propionate pellets into DW and PO are not shown here.

FLOCKS WITH NO PERMANENT MEMBERS

During the second year of observations all the members of flocks 6 and 8 were absentees from those flocks. Previous to the start of rotations the flocks had been organized for at least 6 months. After a 2-week control period, during which the behavior and hierarchy of the members were observed, each member, together with a partner, was introduced into a new flock or, in the case of the hens of flock 6 only, into a small observation pen. For the following 11 months the members of flock 8 met together as a group for only 1 hour on alternate days, while for 6½ months those of flock 6 met together daily for 1 hour. The hens were fed scratch food and observed for the first 10 minutes they were together.

The members of flock 8 became and remained assimilated in the four flocks housed in the henhouse and retained assimilation in their "home flock," as well, throughout the experimental rotations. When brought together as flock 8, the members reacted apparently no differently from members of flocks continuously together, although the proportion of pecks delivered by the alpha hen, Y, increased to a very high percentage of the total (Table 10). Thus it is apparently

possible for a flock to retain its organization even though all its members meet together as a group for only an hour on alternate days.

The only member of flock 6 to become assimilated in both the new flocks into which it was introduced was the alpha hen, BY. Four of the others became assimilated in one of the flocks only, while LW apparently did not become assimilated in either of them. During the last month of rotations, shortly after the change of schedule so that each member of flock 6 spent approximately one-third of its time in either of the two flocks 5 or 7 or in an observation pen, not only did the assimilated individuals seem to lose assimilation in those flocks, but the flock organization of flock 6 was also affected. Thus the individuals tended to avoid each other and to scatter when placed in the pen. They did not feed together, but individually. The proportion of pecks delivered by the alpha hen fell to less than one-third its previous level (Table 11), reflecting the fact that it appeared less aggressive or that the others tended to avoid it more. That the alpha and beta members of flock 6 were the last two hens to be rotated into the condition of partial isolation might be a significant factor. The other factors possibly important in loss of assimilation of these hens have been described elsewhere; and certain of them, such as the physiological changes possibly attendant on their ill-health and their conditioning to being fed in the small observation pen, might have affected the integration in flock 6. The loss of flock integration was not caused by the short amount of time that the members were together, because the hens of flock 8, together for only half the amount of time, were able to retain the appearance of a normally organized group.

TABLE 11

PECKS DELIVERED AND RECEIVED ACCORDING TO RANK IN FLOCK 6
(Figures are in percentages of the total pecks for a given period*)

Dates†	BY (Rank 1)		BR (Rank 2)		RY (Rank 3)		RL (Rank 4)		LW (Rank 5)		BW (Rank 6)	
	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.	Gave	Rec.
First 2 weeks.....	20	.....	60	14	.....	60	20	7	.....	7	.....	13
Oct. 23–Dec. 21...	52	.....	29	17	12	25	2	10	5	24	.....	23
Dec. 22–Jan. 28...	45	.....	21	14	15	16	16	20	3	24	.....	26
Jan. 29–Apr. 10...	35	.....	10	29‡	23	9	6	15	27‡	36	.....	11
Apr. 11–May 8...	11	.....	7	11	43	6	9	25	31	24	.....	34
7-Month total.....	42	.....	20	19	18	17	8	15	13	28	.....	21

* Hierarchy ranks refer to Table 9.
† Dates refer to periods shown in Table 3.
‡ LW became dominant to BR after a short fight on March 25.

Apparently, certain hens cannot adapt themselves to the changes and rotations of this type of experiment. Whether this is because of individual differences in temperament, adaptability, or health or because of the differences in housing and technique is difficult to establish. Other similar hens, however, which are together for as short a time as an hour on alternate days can maintain a flock organization.

RECOGNITION

There is much evidence that the underlying factor in the dominance-subordination type of social organization is the ability of each hen to recognize and

remember each flockmate as an individual (Schjelderup-Ebbe, 1922, 1935). This factor was therefore tested over a period of 2 months at the end of the first year of observations. Each resident and part-time member met each other member of its particular flock in a confined area on territory unfamiliar to both. There were 128 such individual staged encounters, of which 9 were between hens which had not previously been placed together. In 6 of the 9 control encounters there was either a fight or some overt dominance or subordination behavior; in 3 there were no apparent results. Of 119 encounters between hens of the same flock, in 31 cases there were no obvious results, while in the remaining 88 cases the individuals reacted toward one another in the same manner as they did when the entire flock was present. Dominants either pecked or threatened subordinates, while the subordinate submitted to the pecking or avoided the dominant hen. Thus apparently the hens were reacting toward each other as individuals. During the first year the members which were in four flocks reacted according to their status toward 26 other hens, while those rotated during the second year apparently recognized 27 other hens. Dr. A. M. Guhl (personal communication) reports that it is probable that the hens in his flocks of 97 individuals recognized one another, although he believes that that number is approaching the limit which a given individual can remember.

TERRITORY

Allee (1942) has stated what other workers with avian social hierarchies have recognized: "All group organizations among birds are apparently based on the ability of the birds to recognize and remember their flock-mates as individuals. When territory enters as a factor, recognition of the individual's territory also becomes a part of the group reaction system." In a series of twelve experiments during the first year the flocks were placed on neutral territory before the return of the part-time members, absentees, visitors, or transients. In each case the returned member behaved toward the resident hens in exactly the same fashion as it had reacted when the group was in its usual pen. In another experiment an entire flock in which the part-time members were subordinate was moved into the pen where the same part-time members were dominant to the hens they usually met in that pen. Then the reverse experiment was also tried. In no case did the returned part-time individuals react differently toward the resident hens from the manner in which they usually acted when the residents were in the usual pen. It is thus fairly obvious that the part-time members were really reacting to the members of the group and not to the territory in which the group was housed.

TREATMENT WITH TESTOSTERONE

Since the experimental use of hormones to determine their effects on the behavior of individuals in social flocks was first suggested by Allee (1936), there has been a growing literature on this topic. Allee, Collias, and Lutherman (1939) reported that the injection of the male hormone preparation, testosterone propionate, into low-ranking birds resulted in their rise in the flock hierarchy. Domm (1927) had shown that hens secrete androgens as well as estrogens; and Witschi and Miller (1939) summarized the evidence that hens normally produce a certain amount of male hormone. Thus the injection of this material did not mean the injection of a foreign substance into the females.

Pellets of 75 mg. of testosterone pro-

pionate[6] were used in order to give the maximum concentration of intensity and duration of effect (Deanseley and Parkes, 1937). One pellet was implanted subcutaneously into each of the two hens, PO and DW from the home flock 8 (Table 7).[7] At this time, PO was omega in four flocks and next to omega in the fifth; and DW had previously lost status in three flocks, probably because of a severe respiratory infection which made breathing difficult; it had recovered from the infection but had not regained its lost status. Within 2 weeks, both hens became more aggressive and started to show the typical behavior described by others (Allee, Collias, and Lutherman, 1939; Davis and Domm, 1941). They frequently flapped their wings and crowed or waltzed toward the other hens with one wing lowered.

Within 10 days DW had become dominant in all five flocks; and PO became dominant in flocks 1 and 3, where it was present at different times from DW, and beta in the other three flocks where DW was present simultaneously. Further details are given in the following section. Thus, apparently, it was not necessary for a member to be continuously present in order to rise in the social hierarchy because of increased aggressiveness induced by the hormone.

DETAILED RESULTS IN ONE FLOCK

It is impossible solely by general and tabular means to portray the complexity of interrelationships which results from this type of experimentation. Consequently, the history of one of the flocks during the second year of observations will be given in some detail. Flock 3 was chosen because it showed fairly typical results and, in addition, presents interesting special features. There were no strong individual differences among the hens which led to triangles in some groups, yet some of the difficulties encountered in this type of experimentation were present.

During the first 2-week control period the flock was observed to have the straight-line hierarchy indicated in Table 12.

TABLE 12

SOCIAL HIERARCHY IN FLOCK 3 BEFORE THE INTRODUCTION OF PART-TIME MEMBERS

Hen	Pecked				No. of Hens Pecked
PG.....	BW	GW	PR	YW	4
BW.....		GW	PR	YW	3
GW.....			PR	YW	2
PR.....				YW	1
YW.....					0

The first pair of transient hens introduced were *B* and *DW*,[8] second and third, respectively, in the hierarchy of the home flock 8 (Table 7). They were introduced to the flock as a group on October 24, 1944, after having been allowed to scratch around the front area of the pen while the resident hens were confined behind the central doors. The residents and newcomers could see one another through the wire of the partition. *DW* fought with PG and GW, and *B* fought with YW through the screens. When the doors were first opened, *B* and PR fought immediately, and PR won. *DW* and PR started to fight; but the resident alpha, PG, interfered and fought with *DW*. PG apparently won. BW, beta resident, pecked *B* and *DW*, GW pecked

[6] The writer wishes to express her appreciation to the Schering Corporation of Bloomfield, New Jersey, for furnishing the testosterone propionate pellets, and to Dr. Elsie Taber for assistance and demonstration of the technique used.

[7] As controls, the other two members of flock 8 were each implanted with a paraffin pellet.

[8] For clarity, each of the three pairs of hens introduced into flock 3 has been set in different type, thus: *B* and *DW;* the second pair, TG and DY; and the third pair, Y and PO.

DW; and the newcomers submitted to these pecks. YW, omega resident, submitted to being pecked by *DW* but fought with *B* until *B* won. When *B* avoided further contact by flying to the roost, PR chased it to the floor, where *B* submitted to PG, BW, and GW. Subsequent observations showed that the results of these initial pair-encounters were not reversed and that there was a straight-line hierarchy, with the newcomers fifth and sixth, as shown in Table 13.

TABLE 13

FLOCK 3 WITH *B* AND *DW* AS PART-TIME MEMBERS

Hen	Pecked						No. of Hens Pecked
PG....	BW	GW	PR	*B*	*DW*	YW	6
BW....		GW	PR	*B*	*DW*	YW	5
GW...			PR	*B*	*DW*	YW	4
PR....				*B*	*DW*	YW	3
B.....					*DW*	YW	2
DW....						YW	1
YW...							0

Within a week *B* started to feed with the resident hens, but *DW* went into a severe moult and was not considered assimilated until the end of November. Frequently, there were no pecks seen during an entire observation period. Before *DW* was considered assimilated it was observed to fight once with PR and once with BW. From December 19, 1944, to January 6, 1945, *B* and *DW* were each in two groups other than flock 3, and from January 6 to March 25 they were each in three other flocks. Throughout this 3-month period they were present in flock 3 for only 1 hour on alternate days. The resident alpha hen, PG, died December 19, but its death did not affect the social relations within the group, and the former beta resident became alpha, and each of the others maintained its relative rank.

From March 25 to April 22 the amount of time spent in three of the four flocks in which *B* and *DW* were members was equalized, so that they now spent approximately one-third of their time in flock 3. On April 5, PR died. Four days later, two new birds, **TG** and **DY** were introduced into the flock. These birds had been together in a small observation pen in the greenhouse since their arrival in the laboratory the previous October. They were introduced in a series of individual encounters with resident and transient members of flock 3, in the front half of pen 3, while the rest of the group were behind the central doors. The wire area of the doors was covered with beaverboard, so that the old members could not observe the fighting. After the newcomers had scratched around the front area of the pen for 1 hour and 25 minutes, the omega hen, YW, was allowed to join them. YW fought with both newcomers and apparently lost to both. After 5 minutes YW was removed and GW brought in. GW immediately pecked both strangers, and both submitted to it. GW was then removed and *B* carried in. **TG** immediately attacked *B* and fought with it, then **DY** fought with *B*, and *B* became subordinate to both. This was interesting because there is some preliminary evidence indicating that, when an individual once loses a fight, the failure tends to condition it to lose successive ones. However, in these encounters **TG** and **DY** first fought and became dominant to YW, then submitted to GW without a fight, then attacked and fought with *B* and became dominant to it. If fatigue were a factor in **TG**'s and **DY**'s not fighting with GW, then they recovered sufficiently in 5 minutes' time to win the fight with *B*.

B was then removed and *DW* brought in. *DW* fought with **DY** until the latter submitted, then with **TG** but with no ap-

parent result. After 5 minutes, *DW* was removed and BW brought in. This hen fought **TG** and finally won dominance over it, then **DY** submitted to BW without a fight. At this point the doors were opened, and all the members of flock 3 moved into the area. Table 7 shows the hierarchy at this time.

Early in May, approximately four weeks after their introduction, **TG** and **DY** were considered assimilated members of the group. Meanwhile, on April 17, two new transients, **Y** and **PO** were introduced into flock 3, but were never present at the same time with *DW* and *B*, and therefore their history in the flock will be considered separately.

After April 22, *DW* and *B* were rotated among five flocks. About this time *DW* developed the severe respiratory infection already mentioned. From the middle of May to the middle of June, *DW* tended to avoid the other hens, and *B* remained near to it. At an an unobserved encounter during this period, *DW* lost status to **DY**, presumably because of the respiratory infection. At the same time, *B* was observed to lose a fight with YW, thus becoming omega. By the end of June, *DW*'s infection was better, it stopped avoiding the others and pecked YW whenever YW pecked *B*.

On August 3 a pellet of testosterone propionate was placed beneath the skin of the body under the left wing of *DW*. By the seventh of the month *DW* had started to fight with all its more dominant flockmates, and by the fourteenth of the month it was dominant to all except the resident alpha hen, BW. It later became superior to BW as well. On first being returned to the pen, *DW* would waltz toward the other hens with one wing lowered, and the flock would scatter in all directions. *DW* fed alone or with *B* at first but gradually allowed the other hens to feed with it.

Thus *DW* and *B* had been members of flock 3 for 10½ months, throughout which time *B* had held a relatively subordinate status, although it was always considered to be assimilated. On the other hand, *DW* gained status when new members were introduced; lost to one of them, probably because of the respiratory infection; and then became alpha transient after the implantation of the pellet of testosterone propionate (Table 14).

The third pair of hens introduced into the flock was **Y** and **PO**, alpha and omega, respectively, in the home flock, 8. At the time of their introduction they were already assimilated in three other flocks. They met the resident members of flock 3 in a series of individual encounters on April 17, 18, and 19. On the seventeenth, **Y** and **PO** were placed together on neutral territory, and YW, resident omega hen, was placed with them. **Y** and YW fought, while **PO** hid in a corner. Finally, **Y** won and chased YW. **PO** avoided the resident. The following day YW was again brought to the strangers on neutral territory. **PO** hid, and when YW threatened it, **Y** pecked YW. YW was then removed and **TG** brought in. **Y** attacked **TG**, but after a fight **TG** became dominant to **Y**, and **PO** submitted without a fight.

TABLE 14

FLOCK 3 WITH *DW* AND *B* AFTER *DW* HAD HAD A PELLET OF TESTOSTERONE PROPIONATE IMPLANTED SUBCUTANEOUSLY

Hen	Pecked						No. of Hens Pecked
DW....	BW	GW	TG	DY	YW	*B*	6
BW....		GW	TG	DY	YW	*B*	5
GW...			TG	DY	YW	*B*	4
TG.....				DY	YW	*B*	3
DY.....					YW	*B*	2
YW...						*B*	1
B.....							0

On the third day of introductions, **DY** was the first hen placed with **Y** and **PO**. Both strangers submitted without a fight. **DY** was removed and YW brought in. Immediately **Y** threatened YW, and the latter tried to leave the pen. YW was removed and **DY** returned. Immediately **Y** and **PO** crawled under the shelf which holds the water trough and hid there. **DY** was again removed, and GW carried in. **Y** and **PO** submitted. When GW was removed and BW taken into the neutral territory, **Y** immediately attacked, and a fight ensued, which BW won. **PO** avoided contact, then submitted to BW and flew to the roost. Thus again it was observed that a newcomer submitted to one hen, threatened a second, submitted again to the first hen, and then attacked a third hen—all within 15 minutes' time.

TABLE 15

FLOCK 3 WITH **Y** AND **PO** AFTER **PO** BECAME DOMINANT

Hen	Pecked						No. of Hens Pecked
PO	BW	GW	**TG**	**DY**	YW	**Y**	6
BW		GW	**TG**	**DY**	YW	**Y**	5
GW			**TG**	**DY**	YW	**Y**	4
TG				**DY**	YW		2
DY					YW	**Y**	2
YW						**Y**	1
Y			**TG**				1

During the fourth day, **Y** and **PO** were allowed to scratch around the front part of pen 3 for a short while. YW was brought in and immediately pecked **PO** hard. Then the separating doors were opened; **DY** entered first, fought with **Y**, and won. The other hens moved into the pen, and the newcomers avoided them. The hierarchy observed during subsequent observations was the one shown in Table 7.

Two weeks after their introduction to this group, i.e., before their assimilation into flock 3, **Y** and **PO** were rotated into another new flock, and thus were becoming assimilated into two groups at once. They spent approximately an equal amount of time in all except the home flock. Within 4 weeks of their introduction to flock 3 they were feeding and mingling with the residents and were considered assimilated members of the group. Thus the fact that they were becoming assimilated into two flocks at the same time did not lengthen the time required for assimilation. When **Y** and **PO** were first brought into flock 3 from other groups, usually no attention was paid to them by the resident hens, and frequently no pecking at all was observed. During the last week in July, **Y** became dominant to **TG**.

On August 3, **PO** had a pellet of 75 mg. of testosterone propionate implanted subcutaneously, as described for *DW*. On August 18, **PO** had a long fight with BW, and from then on fought daily, until gradually it became dominant to all resident hens and also to **Y**. At the same time that **PO** achieved dominance over **Y**, the subordinate resident, YW, also became dominant to **Y** (Table 15). When the transients were first brought into the pen from another flock, the residents avoided **PO** as it crowed and waltzed toward them with one wing lowered, but gradually they paid no attention to it.

Thus **Y** and **PO** had been present for almost 5 months by the end of the experiments and had held a relatively subordinate status in the hierarchy throughout until **PO** received an implanted pellet of testosterone propionate and rose in the social hierarchy.

It is interesting to contrast the cases just cited in which there were minor shifts in the social hierarchy, such as *DW*'s loss to **DY** probably because of her infection and **Y**'s sudden dominance over

TG, to the order in flock 4 where none of the four part-time members changed status from the time of their arrival to the close of the experiment, except when **PO** received the pellet of testosterone propionate. Thus the hierarchies shown for that group in Table 7 were constant for periods of time varying from 4 to 9 months.

DISCUSSION

IMPORTANCE OF FLOCK ORGANIZATION

The importance of flock organization and of assimilation of each individual to the members of the group, as well as to the group as a whole, was discussed by Allee (1940) and has recently been investigated by Guhl and Allee (1944). They reported that in the organized flocks composed entirely of assimilated members the individual hens showed reduced fighting, increased food consumption, better maintenance of body weight, and higher egg production, as compared with the hens in flocks continually undergoing organization. They concluded that the flock organization which results from individual competition together with learned toleration gives a co-ordinated social unit which is better fitted for survival than the same group of individuals would be were they fighting among themselves. Thus competition at the individual level helps to give rise to an organized group which is better fitted to co-operate as a social flock. Collias (1944) presents evidence of similar nature.

REACTIONS OF THE GROUP TOWARD STRANGERS

The reactions of the individual members of organized groups toward strangers which prevent the newcomers from entering the group have been reported for various vertebrate groups in the laboratory, as well as in the wild or semi-wild conditions (Braddock, 1945, and Greenberg, 1947, for fish; Carpenter, 1942, for rhesus monkeys; Hamerstrom, 1942, and Odum, 1941, for chickadees; Howard and Emlen, 1942, for valley quail; Guhl, 1945, for small flocks of domestic fowl). Guhl and Allee (1944) have shown that new members of a flock of White Leghorn hens are usually dominated by the members previously present.

Braddock (1945) reported that in all but two of his introductions the highest-ranking fish attacked the strange newcomer first. This is similar to results described with the hens in which the dominant hen was the first or the second resident to fight with one or both of the newcomers or interfered in fights when a subordinate resident attacked a newcomer first. Whatever the reason for the alpha individual's starting the fights—and possibly it is that the highest-ranking bird is usually (although not always) the most aggressive of the group—it is obvious that the animal in alpha rank stands to lose most if the newcomers were to become dominant to it.

Shoemaker (1939) states that, although a strange canary introduced into flocks of that species might win the first few fights, it is ultimately weakened, being outnumbered by the resident birds, and others come to dominate it in a short while. This is consistent with a statement by Schjelderup-Ebbe (1922) that fighting a single hen over and over eventually weakens it so that it loses to relatively weaker hens. To avoid this factor, the two newcomers in thirteen cases were matched with each resident hen individually before meeting the group as a whole. In these cases the results of the pair-encounters were maintained after the strangers met the residents as a group. The important fact is that, in spite of the obstacles to becoming a member of a strange group, in 43 out of 53 introductions the strange hens were able to become assimilated (Table 4).

FACTORS AFFECTING ASSIMILATION OF NEWCOMERS INTO ORGANIZED GROUPS

Assimilation time.—The length of time required for assimilation was apparently not affected by (1) the number of flocks in which the individual was already assimilated, (2) the method of introduction into the new group, or (3) the previous history of the individual. The most important single factor influencing the time for assimilation was the status which the hen achieved during the initial encounters with resident hens. As noted in the previous section, newcomers in the alpha, beta, or gamma positions were assimilated within 3 days of introduction, while others required from 3 to 6 weeks to become adjusted.

Physical condition of the newcomers.—Schjelderup-Ebbe (1935) outlined the factors which determine the hen's initial status in the hierarchy, and these have been partially statistically analyzed by Collias (1943) for initial pair-contacts. Collias reported that, in individual pair-encounters of hens under the conditions of his experiments, 44 per cent of the factors deciding the outcome were undetermined, while of the remaining 56 per cent the most important were: (1) the slightness of moult; (2) the amount of male hormone as indicated by the size of the comb; (3) the social rank of the individual in its home flock; and (4) the weight (assuming no very large differences). In the work at hand no individuals were moved into a strange flock while the newcomers were moulting. During the first year experimental hens with relatively large combs were selected for rotation, but during the second year the entire membership of two flocks were used, regardless of comb size. To the observer all the part-time members were healthy and normal at the start of the experiments.

The influence of conditioning.—The role of conditioning in these experiments is difficult to ascertain. It has already been noted that the members which were relatively dominant in other groups tended to fight harder during the initial encounters, while the relatively subordinate individuals would frequently submit or avoid without a fight. It is of interest that in the detailed results of pair-encounters described in flock 3, newcomers might fight one strange resident paired with them; after that, submit to a second without a fight; and then fight a third resident within 15 minutes' time. If conditioning were operating strongly here, one would expect that, after winning the first fight, the hen would fight a second individual brought into the territory. This was apparently not the case, perhaps, in part, because of fatigue.

Guhl and Allee (1944) found that alternate hens which were moved from flock to flock (instead of from the isolation pen to the flock) displayed learned social reactions when moved in steady succession between flocks which caused them to fit into the new social levels with less time for adjustment than was needed by birds which were removed from isolation and placed in an unorganized group. Thus the previous history of the hen does in some way condition it to the experimental procedure. However, the whole process of assimilation depends not only on the adaptability of the newcomers but also on the reactions of the resident hens toward them.

Territory.—One of the points of difference between the assimilated hens and those which failed to become or to remain assimilated was that the latter spent one-third of their time in the confines of a small observation pen. It is possible that they became conditioned to feeding in this confined space. The partners which were together in the area

seemed to show increased toleration for each other when returned to the home flock, for they tended to remain close together. Allen (1934) in work with ruffed grouse found that, when in an inclosed cage, one bird was "subjugated" by the attacks of a larger companion, it was then subjected to attack from every other bird in the inclosure. The completely subjugated bird "developed an *inferiorism* and usually, unless removed, he remains in the corner until he dies . . . not from mechanical injury nor from starvation, but from some sort of nervous shock, and death is likely to occur within twenty-four hours" (p. 183). It is possible that the four members of flock 6 which became ill and died may have become ill from the effects of the rotations and thus have lost assimilation or that the rotations might have caused some psychological disturbance, such as Allen has discussed, which made it impossible for them to become assimilated or to retain assimilation in the new flocks. From the few cases at hand it is extremely difficult to evaluate the many factors which might have been responsible. Of course, in accord with Lloyd Morgan's well-known canon, it is best to avoid undue emphasis on unexplained psychological factors.

INTRA-FLOCK RELATIONS

The assimilated part-time members apparently had little if any effect on the relations between resident hens. Throughout approximately eleven months during each of two consecutive years, the social hierarchies of the resident hens remained stable with the one exception noted above (p. 164). This can be correlated with the observed behavior, which was apparently no different when part-time members were present than it was when they were not. Some indications of this similarity of behavior can be obtained from the figures in Table 8. For example, the alpha resident of any one flock delivered approximately the same proportion of the total pecks whether subordinate part-time members were present or absent. If the latter were dominant, however, the pecking of the alpha resident tended to decrease to the level of the beta hen. Comparisons of the percentages given and received by other members of the hierarchy show the same order of magnitude, whether the resident hens were alone or with part-time members. Obviously, when part-time members which were subordinate were present and received a share of the pecks according to their rank, the percentage of the total given to residents dropped somewhat, because the total pecks were divided among a greater number of hens. For example, the fifth resident in flock 1 received 56 per cent of the pecks when no part-time members were present, but only 33 per cent or 30 per cent when the different pairs of transients were in the pen. Similar results can be seen in the other flocks shown.

On the other hand, the presence of nonassimilated members somewhat affected relations among the resident hens because at times all pecks were delivered to those unassimilated individuals. Subsequent observations on the resident members showed that their social hierarchy was unaffected.

Just as the presence or absence of assimilated individuals appeared to have no effect on the relations among the resident hens, so the former were treated, when present, in the same manner as any resident hen with similar status. Thus if relatively dominant part-timers were placed in the pen, the resident hens submitted and avoided, while, if subordinate ones were present, the resident hens pecked them and the part-timers submitted and avoided. Assimilated part-

time members apparently received neither higher nor lower proportions of the pecks than did resident hens with similar social status. This shows, first, that they were not pecked discriminatorily and, second, that they did not avoid the pecking any more than did resident hens. When the wide variations in aggression and submission are considered, the fact that these percentages of the total pecks among residents and assimilated part-time members are in most cases so similar is a strong indication that the part-time members were treated in the same manner and reacted in similar manner to hens permanently present.

There were differences from flock to flock in the tendencies of high-ranking birds to allow lower-ranking individuals to feed with them. Thus in some flocks subordinate hens were kept on the fringe, while the dominants fed first. This did not necessarily indicate that these subordinates were not assimilated, since, as has been shown, there are several factors involved in the process of assimilation.

The main difference between a subordinate assimilated individual and an unassimilated one was that the former tried to feed with the group and usually mingled with the group in its other activities, such as dusting, etc., and did not usually try to avoid an approaching resident hen. The behavior pattern of the unassimilated hen was one of apparent caution and possibly fear, so that sometimes the slightest movement of another individual would cause it to run rapidly around the pen. This activity would stimulate the residents frequently to peck the hen as it ran by, sometimes to chase it as well. A hen which was becoming assimilated gradually lost these characteristics and came to behave as one of the flock, for it tended to feed, dust, and join in the other activities of the group more and more. Thus there are, within a given rank, various levels of assimilation during the assimilation period; and notably in the case of an omega individual there are apparently several subranks which are evident and which gradually grade off into a completely nonassimilated individual.

Intra-flock relations in groups with no permanent members have been presented in the previous section. The members of flock 8 which were placed together as a group for 1 hour on alternate days retained for a period of 11 months of observations all the characteristics of the typical flock hierarchy. It should be noted that each member was present in other flocks with one partner from the same home flock, so that all were continuously in the presence of at least one flockmate. Similarly, the members of flock 6 retained flock organization for the first $5\frac{1}{2}$ months of the rotations. There was one apparent difference, however, between the two flocks: the two hens of flock 6 which spent their time in a small observation pen tended to remain closer together than did any of the others. This could perhaps be interpreted as evidence of increased toleration of these hens for each other.

EVIDENCES OF CO-OPERATION

Several evidences of co-operation were especially marked between partners which were moved into new flocks together. There was apparently no pecking by the dominant of the two partners before both hens became assimilated into the new group. After assimilation one partner pecked another only rarely.

When one hen of a transient pair was dominant to a resident, frequently it would peck the resident whenever the latter pecked its partner. Thus, for example, DW or B would peck any resident which pecked PO in flocks in which they were partners with PO and dominant to

residents (Table 7, flocks 2 and 4). Similarly, RG pecked GY whenever GY pecked WW when the partners, RG and WW, were returned to the home flock, ii (Table 2). Similarly, RW pecked the resident hens of iii, which pecked its partner, BB, although BB was dominant to RW and occasionally pecked it.

Nonrotated hens which were introduced together into a new flock as permanent members also tended not to peck each other. For example, only occasionally did TG peck DY (flock 3, Table 5), although it frequently pecked other hens to which it was dominant. This is similar to the results reported by Vogel (1944), in which he introduced two young chicks together into a group and they tended to form a social unit without pecking or fighting each other.

POSSIBLE EFFECTS OF FRUSTRATION

The term "frustration" has been variously defined. Seward (1945) uses the definition given by Dollard, Doobs, Miller, Mowrer, and Sears (1939), that frustration is interference with a goal-response, a "goal-response" being defined as an act which terminates and reinforces a behavior sequence. Dollard *et al.* state that "aggression is aways a consequence of frustration." Scott (1946) defines frustration as the prevention of a successful adjustment to a change. Scott considers that the reaction to frustration might be either incomplete adjustment or nonadjustment.

Seward designed a series of experiments to test the hypothesis that aggression is always a consequence of frustration in white rats and concluded that frustration is not the only cause of aggression but that another possible factor is the stimulation from a strange animal of the same species. From the work at hand, there are numerous examples given in which stimulation from strange hens has resulted in expressions of aggression. Scott concluded that frustration of inexperienced fighting mice results in incomplete adjustment. It is difficult to ascertain whether any of the reactions discussed herein could be considered incomplete adjustment, unless one could consider the individuals which did not achieve assimilation as incompletely adjusted to the conditions of rotation or to the new flocks in which they were placed. It is far more probable, however, that the nonassimilated individuals were not at all adjusted, a result which Scott has stated results in abnormal behavior. It has yet to be proved that this lack of adjustment was a result of frustration of the individual hens.

There are certain other cases in which the aggressive responses might be considered the results of frustration, since they were apparently not elicited by the presence of strange hens. For example, both alpha transients in flock 2, DW and B, delivered more than 50 per cent of the total pecks (Table 8), and simultaneously both were subordinate in other flocks which might have caused them to become frustrated, assuming the definition of frustration as interference with a goal response and again assuming that their subordinate position interfered with goal responses. Both these hens similarly delivered a higher proportion of the total pecks in flock 4, where DW gave 34 per cent of the total and B gave 45 per cent. These variations may be no higher than individual variations might allow, or they may be evidence of increased tensions. It is possible that Y was frustrated because of holding a subordinate status in three flocks and therefore, when in the home flock, 8, delivered a far higher proportion (up to 90 per cent) than is usually given by an alpha hen (Table 10). If it were frustration alone, however, which caused this show of aggression, it is dif-

ficult to explain why the same hen, dominant in flock 4, delivered only 22 per cent of the total in that group (Table 8).

Alpha resident hens tended to deliver the same proportion of the total pecks when no part-time members were present as when subordinate ones were with the group. When alpha transients were present, the number of pecks delivered by the resident alpha hen fell to the level of that usually delivered by a beta hen. If these hens were frustrated by the presence of the alpha transients and if aggression followed frustration, one would expect them to deliver a higher proportion of the pecks at that time. That this was not the case may indicate (1) that the transient alpha hens' presence did not frustrate the alpha resident or (2) that, if the alpha resident was frustrated, this frustration was not followed by any evidences of increased aggression. The whole matter of frustration in nonhuman animals needs to be treated with marked caution.

POSSIBLE EVOLUTIONARY IMPLICATIONS

Wright (1940) has shown that the populations which are subdivided into small local populations which breed among themselves but receive a proportion of migrants which interbreed with the local residents are the most likely to show most rapid evolutionary changes and are most likely to be able to adapt to environmental changes. The difficulties encountered by strangers in becoming assimilated into organized groups of many vertebrate species have been discussed. In spite of these difficulties, however, Odum (1941*b*) found that certain chickadees could become members of new groups. These migrants allow for genic recombinations in otherwise isolated groups. Thus the group-survival values, such as those shown by Guhl and Allee (1944) for small flocks of hens, can continue to operate for the group, but without causing the group to become completely isolated, a condition which Wright (1940) has shown may result in the fixation of the genes. Collias (1944) suggests that evolutionary trends in aggressive behavior are related to evolutionary trends in social phenomena. Thus the entrance of strangers into organized groups can result in greater numbers of gene variations and in this way be of significance to the entire species population.

SUMMARY

1. It is possible for a hen to become an assimilated member of as many as five different flocks and simultaneously maintain a different status in the hierarchy of each.

2. Assimilated members present in the group for only part of the time reacted toward flockmates in the same way as hens with similar social status which were permanently present.

3. Resident hens reacted toward assimilated flockmates in accordance with their social status, whether the latter were present permanently or only at intervals.

4. Assimilated part-time members of the group received and delivered approximately the same proportion of the total pecks as did resident hens with similar social status.

5. The factors which affected the assimilation of newcomers into organized groups may have included health, conditioning, territory, recognition, and the degree of aggressiveness on the part of the newcomers in attempting to mingle with the resident hens.

6. Newcomers which achieved alpha, beta, or gamma rank apparently became assimilated within 3 days of their introduction; newcomers which did not achieve relative dominance required from 3 to 6 weeks.

7. Some individuals were unable under certain conditions to become or remain assimilated.

8. Unassimilated individuals were not allowed the freedom of the pen and were frequently chased by the resident hens; they tended to avoid the other members of the group.

9. In only one case did the introduction of part-time members into an organized flock apparently affect the hierarchical relations of the resident hens; in the twenty-one other cases the introduction or removal of rotated pairs of hens did not affect the status of the residents.

10. The relations among resident hens of a flock were apparently unaffected by the presence of assimilated part-time members. These relations were somewhat altered, however, by the presence of unassimilated hens, some of which, under certain conditions, received all the pecks delivered throughout an entire period of observation.

11. It was apparently possible for a social group to remain organized when it was composed entirely of absentee members meeting together in the home group for only half an hour on alternate days.

12. There was evidence of some cooperation between partner-hens which were moved together into strange flocks.

13. The hens reacted toward each other according to rank in the same way, regardless of the territory in which they were placed.

14. Two hens, each implanted with a pellet of testosterone propionate, rose in the social hierarchies of each of the five flocks in which they were members.

15. It is possible for a hen to recognize and react according to rank to a total of twenty-seven other individuals, although this may well not be the upper limit.

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NEW BOOKS IN PHYSIOLOGICAL ZOÖLOGY

[This section comprises reviews of selected new books containing new and significant material in the field of physiological zoölogy. Books submitted for review should be sent directly to the review editor: Frank A. Brown, Jr., Department of Zoölogy, Northwestern University, Evanston, Illinois.]

BIOELECTRIC FIELDS AND GROWTH[1] BY E. J. LUND AND COLLABORATORS

Reviewed by GORDON MARSH
State University of Iowa

THIS important contribution to the literature of biophysics is not a general review but consists of experimental data (unpublished previously, save for abstracts) centering around the senior author's thesis, formulated in 1925, that continuously maintained, self-generated electrical fields operate as primary correlating mechanisms in cell and tissue systems lacking nervous and circulatory devices. The most important example of cell correlation is the control of the growth pattern in embryonic systems.

About one-fifth of the space is devoted to experiments demonstrating the effects of externally applied electric fields upon polar growth. Reversible inhibition of growth of the onion root was found when (apicobasal) potential gradients of 38 mv/mm were applied to the entire root or when about twice this field strength was applied to the apical region alone. No growth acceleration could be demonstrated. Applied currents of appropriate strength tended to increase the rate of elongation of *Avena* coleoptiles when passed from apex to base. Currents applied transversely to the coleoptile apex produced bending toward the positive pole. This response cannot be explained by electrical transport of auxins. Apicobasal applied currents reduced or suppressed the curvature response to lateral illumination or to gravity. Reassociated cell masses of *Obelia* regenerated in an electric field substantially as do cut internodes.

The remainder of the book is concerned with the more exacting, less spectacular aspects of the problem: the distribution of potential differences in space and time and in relation to environmental factors; the effect of applied currents upon the electrical characteristics; the capacity of cell systems to perform electrical work and the efficiency of the process. The importance of experimental data of this type cannot be overemphasized, for the ultimate analysis of the nature of the control exerted over growth and differentiation by externally applied electric fields depends upon an intimate and detailed knowledge of the effects of the field upon the electrical and other physiological properties of the growing system. If such knowledge is to be applied to the understanding of the self-generated, self-limited morphogenetic fields operating in normal development and to the test of their physical reality, there is demanded, in addition, the fullest possible information of the spatial and temporal distribution of the self-generated electrical potential differences within the organism and of the patterns and limitations of internal electrical-energy expenditures.

Meticulous measurements were performed on the distribution of potential over the surface of *Pithophora*, a single, linear cell aggregate, over and within the mantles of *Anadonta* and *Unio* and the coleoptile of *Avena*. A thoroughgoing study was made of the spontaneous variations in inherent potential difference in the onion root with constancy of all external conditions, and of the distribution in magnitude of the variations over the root. The effects of gravity upon the inherent potential distribution in the *Avena* coleoptile appeared in characteristic pattern (underside of apex positive to upper) 20–30 minutes before curvature could be detected. The relation of these facts to hormone action is discussed. The counter-E.M.F. developed in the onion root by applied currents was carefully studied in relation to current intensity, duration, direction of flow, region of the root, and effects of oxygen tension and cyanide. The threshold of detectable polarization was again found to fall within the range of the root's self-generated current. The authors conclude from the data that three electrochemical systems are present in the root: a polarizable system con-

[1] Austin, Tex. University of Texas Press, 1947. Pp. vii+391. $6.00.

sisting of free ions distributed within and without the cells; a nonpolarizable system generating electrical energy continuously (oxidation-reduction); and a nonpolarizable, metastable system, which may respond by a decrease in root potential independent of the direction of the applied current. (The evidence for the separability of these systems appears inconclusive to the reviewer and the nonpolarizability of the second to depend upon hypothesis alone.)

The "over-all" efficiency of frog skin as an electrical generator was measured (microcoulometer, potentiometer, and milliammeter) by comparing the electrical energy delivered through a low-resistance external circuit to the energy equivalent of the oxygen consumed during the same interval (Winkler method). An average value of about 1.5 per cent and a maximum of about 5 per cent were found. An interesting quantity defined as the "coulomb efficiency" was obtained from the ratio of total coulombs delivered by the skin to the coulomb equivalents of valence change in the consumed oxygen. Values of from 30 to 60 per cent were found. While the entire significance of the "coulomb efficiency" is not clear, it evidently represents a test of the capacity of the oxidative mechanisms of the skin to maintain continuous current flow. The current intensity, its rate of decline with time, and its effective duration were shown to depend upon the oxygen tension of the medium and to be reduced by cyanide.

The experiments were thoughtfully conceived and carefully executed and, in the opinion of the reviewer, offer strong support to the major thesis. Methods are described in detail. Throughout the book the authors are careful to separate experimental results, legitimate inference, and free speculation. Seventy-four figures, ten tables, and several experimental protocols afford adequate documentation. One hundred and eighty references are cited. The short—too short—general discussion of the significance of the results appearing at the end might profitably have been expanded into a complete theoretical and experimental analysis of the problem, synthesizing present data. The few typographical errors and a mistake in one text diagram are unimportant. A valuable addition to the book is the Bibliography of fourteen hundred and six titles on continuous bioelectric currents and bioelectric fields by Dr. H. F. Rosene, which is beautifully cross-indexed as to subject matter, method, and materials.

HEREDITY AND VARIATION IN MICROÖRGANISMS ("COLD SPRING HARBOR SYMPOSIA ON QUANTITATIVE BIOLOGY," VOL. 11)[1]

Reviewed by ADRIAN M. SRB
Cornell University

VOLUME 11 of the "Cold Spring Harbor Symposia on Quantitative Biology" presents a series of papers dealing with various aspects of the subject "Heredity and Variation in Microörganisms." The topic is timely and the individual articles are most thought-stimulating, both because of the information presented and because of the interpretations correlating recent knowledge with that longer established.

The bulk of the symposium deals with problems of heredity in molds, bacteria, and viruses. Throughout, one can perceive the highly desirable tendency in modern genetic investigation to utilize and appreciate various aspects of the organism which once were held to be provinces of particular compartments of knowledge and research. Conspicuous in this regard is the ever closer relationship between biochemistry and genetics. In many ways the symposium reflects the fruitfulness of the biochemical approach to problems of heredity.

It is significant that more than a few of the papers deal with heredity in forms in which it has not been clearly established that sexual reproduction occurs. Such studies, in a way, represent instances in which workers have forsaken the standard genetic approach by way of segregation and recombination of hereditary units. In modification of this impression, however, it should be said that it now appears that recombinations of characters may occur among mixed populations of certain bacteria. Evidence that this is so is presented in a paper by Lederberg and Tatum; and other papers in the symposium provide pertinent background material

[1] Cold Spring Harbor, N.Y.: Biological Laboratory, 1946. Pp. xii+314. $6.00.

to the important question of whether bacteria have a sexual stage.

In any case the symposium serves to point out a shift in the interest of numbers of geneticists to problems concerning the nature, functions, and mode of action of various kinds of hereditary units. Perhaps such problems could become clearly defined only after that period of classical genetics in which attention was largely centered on the mechanism of transmission of genes in different kinds of genetic systems. With a new era in genetics, however, the field becomes of more interest to others as well as to its own specialists and should be attractive to all biologists, at least in so far as it provides useful points of view for studying what organisms are and how they work. It should probably be emphasized, though, that the older methods of genetics have been supplemented rather than overthrown. That the old genetics and the new can stride hand in hand with extremely felicitous results is shown, for example, in the Neurospora work, in which studies of gene action are amplified by studies of the segregations of the genes.

Geneticists, however, are no longer solely concerned with genes on chromosomes. This is reflected in a series of papers on extra-chromosomal inheritance, including another instalment of the fascinating *Paramecium* story and also a summary and interpretation of the evidence for "cytogenes" in yeast. The problems in these kinds of study are obviously related to questions regarding the autonomy of another kind of cytoplasmic unit, the plastid. This last subject is dealt with in a paper by Rhoades. Most of the work he reviews has been done with higher plants, which helps to stress the fact that many of the basic problems in genetics are fundamental to all living forms and that these problems must be attacked wherever best they may. Thus there is nothing incongruous about papers dealing with certain aspects of cancer being included in a symposium on heredity and variation in microörganisms.

The catholic activities and interests represented in the symposium, in addition to the record of accomplishment, cannot but arouse optimism for the future of research in the life-sciences. It is probably appropriate, however, that the final paper in the published volume is, in effect, a temperate warning that the millennium in the biological field has by no means been reached. In this paper Professor van Niel deals with some of the perplexing difficulties in bacterial classification. He leaves no doubt that there remains a great and profound array of problems to be dealt with in coming time.

A few words may be said about the editing of Volume II of the symposia. Certain selected comment by symposium members is again printed in conjunction with individual papers. Mere matters of bulk and space seem to preclude the printing of the entire commentary, but even that which has been included is well worth while. The reviewer feels strongly that inclusion of some discussion material is stimulating to the reader and is valuable in clarifying matters of interpretation.

The papers in Volume II are published in a sequence that is alphabetical according to the names of the authors. The stated purpose of this kind of arrangement is to provide for greater ease of reference. For no kind of reader, however, does such a system seem to offer large advantages over a situation in which papers are arranged topically and are accompanied by clear and thorough indexing. And, on the other hand, it may be that the pretensions of the publishers of this work are too modest, since it would appear that many persons will wish to read all or large parts of the volume in the interest of better orientation in a rapidly moving field. For such an end there are apparent advantages in a more logical order by way of subject matter.

HORMONES AND BEHAVIOR: A SURVEY OF INTERRELATIONSHIPS BETWEEN ENDOCRINE SECRETIONS AND PATTERNS OF OVERT RESPONSE[1] BY FRANK A. BEACH

Reviewed by W. C. ALLEE
University of Chicago

IN A brief Foreword Earle T. Engle emphasizes the encouraging development of the hormonal phase of comparative psychology into a behavioral physiology. The present book, summarizing this trend, was written when Beach was chairman of the Department of Animal Behavior at the American Museum of Natural History before he moved to Yale University to become professor of psychology.

The author, who refers to himself as "the reviewer," explains in the Preface that he presents the volume as "the expanded and perhaps unwieldy outcome of my own curiosity as to what others have learned regarding the effect of endocrine secretions upon the behavior of animals, including man." The role of the book is said to be "not the exposition of a thesis but the supplying of a body of facts and references." It is intended to be an inanimate tool rather than a living presentation. Beach says that in the course of the work a few major trends emerged incompletely and dimly from the confusing array of factual data drawn from the reports of a great many experiments by a multitude of workers.

The major topics presented are: courtship and mating; reversal or bisexuality of mating behavior; oviposition, parturition, and parental behavior; migration; generalized aggression, social dominance or submission, and territory defense; emotion; conditioning and other types of learning; general locomotor activity; homeostasis, metabolism, metamorphosis, and moulting; morphologic structures employed in specific behavior patterns; the role of nervous stimulation; developmental aspects; major sources of variability; and, finally, interpretations of hormonal effects. There is a long Bibliography, a comprehensive Glossary, and a subject, but not an author, index. The bibliographic citations are predominantly based on work done in the United States and are drawn mainly from the last two decades.

The treatment follows a fairly definite trend. Typically, there is an opening, orienting statement of a few sentences. This is followed immediately by a very brief review of each of a skilfully selected series of papers. These usually deal only with the vertebrates arranged in the customary ascending scale of taxonomic classes, closing with some of the evidence concerning human behavior. The discussion of a given point may or may not be summarized at the end of a section or chapter.

Throughout the book much attention is paid to sexual behavior, with the recurring emphasis on primary mating reactions. In the opening chapter on "Courtship and Mating," for example, the accent is on mating and its underlying physiology rather than on courtship. The approach to human behavior is shown by the following quotation:

> We come now to a point at which some account of the evidence dealing with human subjects is imperative. There are those who would exclude this material from serious consideration, or would at least hesitate to compare it with data derived from studies on the lower animals. Others might feel no reluctance to combine human and animal data, and would subject them to more or less the same interpretations. To the reviewer [Beach] neither extreme view seems justified; and subsequently the more obvious similarities and differences between the results gathered from human and animal sources will be discussed. For the present we shall merely cite a few representative studies which purport to show that the sexual activity of humans bears a demonstrable relation to gonadal condition [p. 11].

In another of his rare personal statements Beach summarizes his thinking about sex reversal and bisexuality:

> The general impression that one gains from a survey of the literature tends to throw some doubt on any concept of sex reversal which depends upon complete sex-specificity both of the behavioral mechanisms and of the gonadal hormones. A somewhat more reasonable hypothesis would seem to be that in many if not all vertebrate species both males and females are equipped by nature to perform at least most of the elements in the overt mating pattern of the opposite sex. In some species, and particularly in the female, the heterologous reactions may occur under appropriate external stimulation without any alteration in the normal endocrine balance. In other instances the copulatory performance of the opposite

[1] New York: Hoeber, 1948. Pp. xiv+368.

sex is activated only by increasing the concentration of heterologous hormones. But even here nothing has been "reversed." Rather, a previously refractory and relatively inactive behavioral system has been rendered more responsive to external stimulation. And be it noted that under such conditions the homologous sex pattern is often simultaneously activated, so that bisexual behavior results [p. 69].

The general concept is that of incipient ambivalence of the behavioral mechanisms of the two sexes.

The description of migration centers about that of birds. Due attention is given to Rowan's work and ideas about migration being controlled by the effect of length of the photoperiod on the gonads. Characteristically, Beach cites supporters and objectors to the idea that gonadal hormones are directly responsible for the migratory performance of many species of birds. This section is ended by outlining Bissonnette's "rather complex theory to explain the initiation and duration of migration," in which attention is focused on an inherent cycle of secretory activity of the pituitary affecting both gonads and the thyroid. "Southward migration follows pituitary regression and continues as long as the refractory phase endures" (p. 94). Beach's method of presenting ideas is illustrated by the citation of A. L. Thompson's opinion that "Rowan's theory suffers from over-simplification" and by Bullough's criticism of the theory of Bissonnette and of Wolfson's multiple-factor explanation of bird migration as being "too hypothetical." Direct critical evaluation is rare in this book.

So far as the present reviewer knows, Beach's treatment of the literature is trustworthy. There is one minor exception that perhaps deserves mention. In discussing generalized aggression, Beach cites (p. 106) the conclusion of Collias (1943) that "androgen and thyroid output are two influences of major importance in determining success in initial encounters between hens." He contrasts this result from a "statistical study" with the experimental finding of Allee, Collias, and Beeman (1940) that "administration of thyroxin is without effect upon the social order of these birds unless a severe moult is induced." Actually, discrepancy, if any, is not so direct. In studying results from staged pair-encounters, Collias found that absence of moult has a higher correlation with winning than has any other observed condition. Since thyroxin induces moulting, Collias reasoned that the factors of major importance in the success of hens in combat were "male hormone output as indicated by comb size and thyroxin as indicated by the complex of changes that accompany moulting" (*Amer. Nat.*, 77:537). Critical discussion might well center on the efficacy of the indicators rather than on the contrast between a "statistical study" of experimental findings and results that did not allow statistical analysis.

There is a temptation to emphasize the occasional references to hormones of invertebrates. To do so would throw this review out of harmony with the organization of the book. Beach states in one place that "additional evidence on invertebrates would be of value." He mentions Haydak's (1943) belief that in the honeybee "both the anatomic and the physiologic differences are due to a great extent to hormones secreted by the fully developed ovaries of the queen." The so-called "social hormones" of ants and termites are not mentioned. Such an omission of pertinent material is an exception rather than the rule.

Although there is much documentation even in the last chapter of the book, Beach finally allows himself more freedom to make direct statements based on his own experience and interpretations. Perhaps it is not unfair to the author to quote briefly his summarizing generalizations. Each of these is discussed with supporting citations.

None of the behavioral responses thus far investigated have been found to depend upon one and only one hormone.

No hormone is yet known which produces one and only one effect upon the organism.

Hormonal control of a complete behavior pattern appears to be mediated by several intervening mechanisms rather than a single critical one.

Different behavior patterns may vary in their degree of dependence upon endocrine products.

Every behavioral response constitutes a reaction of effector mechanisms to internal and external stimuli; the effector mechanisms are neuromuscular or neurohumoral units whose functional characteristics are determined jointly by genetic constitution, by previous excitation, and by the chemical nature of the internal medium.

The potential mechanisms of hormonal control are given as being through their effects on (1) the organism as a whole; (2) specific morphologic structures employed in particular response patterns; (3) peripheral receptor mechanisms; and (4) the integrative functions of the central nervous system. The problem concerning the means whereby observed results are

mediated is raised and discussed, although "present knowledge offers no direct solution" (p. 273).

This book is a useful guide to the literature in the overlapping fields of hormone physiology and animal behavior. It can be recommended heartily both to beginning and to mature students who are working at or near the border line between these two interesting and important phases of animal biology.

TRACE ELEMENTS IN PLANTS AND ANIMALS[1] BY WALTER STILES

Reviewed by DANIEL MAZIA
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BECAUSE the elements which are required by living cells only in minute amounts (e.g., 10^{-6}–10^{-7} molar in nutrient solution) present such an intriguing problem to the biologist who is not a plant physiologist or pathologist, he may be somewhat disappointed in Stiles's little book. But the enormous amount of specialized information that is included should only sharpen his interest in the broader import of the problem. Stiles treats rigorously the criteria of essentiality of inorganic micronutrients. It is clear that the list of essential elements is not complete. Of the twenty or so elements found in small amounts, the list of those established as essential probably includes only Zn, Cu, Mn, B, and Si. Still others, such as Co, Ni, Al, and Mo, often have favorable effects on growth, although they may not have been established as essential. Other elements seem to have very little physiological effect, even though the plant does not exclude them. For the plant physiologist, Stiles's work offers extensive tabulations of micronutrient requirements by species and by element. Stiles's discussion of techniques will provide a good basis for the zoölogist who wishes accurately to control the mineral composition of his media. A considerable part of the work is devoted to detailed descriptions of deficiency symptoms and symptoms resulting from excess of micronutrients. The zoölogist may not be specifically interested in these; but, if an analogy may be made with vitamin research, the study of symptoms may provide essential clues to the investigation of mechanisms. It is striking, for instance, that so many symptoms of trace-element deficiencies in plants concern the plastids.

The weakest point in Stiles's book is his discussion of the mechanism of action of trace elements. He does mention a few older studies of metal-protein enzymes, but a thorough treatment would show that there is, in fact, a fair amount of information on the role of low concentrations of heavy-metal ions in enzyme reactions. There is little discussion of the concentration of trace elements in the cell. Yet there is some reason to believe—and some of Stiles's tables bear on this—that the elements which are present only in trace concentrations in the medium may actually accumulate to much higher concentrations in the cell (cf. Mazia and Mullins, *Nature*, Vol. **147** [1941]). A reasonable approach to the understanding of trace metals probably would involve consideration of the cell's capacity to accumulate them and the concentrations of molecules, such as enzymes, which combine with metallic ions to form active compounds. But, while it would not be difficult to outline a practical approach to the study of metallic ions, the function of nonmetals, such as B and Si, would hardly seem to be so simple.

Reading Stiles's work from the standpoint of the zoölogist, one is particularly impressed with the possible applications to protozoan nutrition and the probable advantages of protozoa over plants for a fundamental study of the problem of the trace elements.

[1] New York: Macmillan Co., 1946. Pp. 189. $3.00.